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INHALATION TOXICOLOGY OF FOG OIL SMOKE

FINAL REPORT

Elaine C. Grose, Ph.D.
Mary Jane Selgrade, Ph.D.
David W. Davies
Andrew G. Stead

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DECEMBER 1986

Army Project Order 1810

Toxicology Branch
Inhalation Toxicology Division
Health Effects Research Laboratory
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, MD 21701

Project Officer: Robert A. Finch, Ph.D.
Health Effects Research Division
U.S. ARMY MEDICAL BIOENGINEERING RESEARCH AND DEVELOPMENT LABORATORY
Fort Detrick, Frederick, MD 21701

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Sixty-day-old, male and female rats were exposed by inhalation to fog oil smoke. Mortality, LC$_{50}$, subacute and subchronic studies were performed. Mortality studies showed fog oil smoke to be 100% lethal at 11.0 mg/L, 95% lethal at 5.0 mg/L, 20% lethal at 1.0 mg/L and 0% lethal at 0.1 mg/L after a 6-hr exposure. The observed LC$_{50}$ of fog oil smoke after a 3.5-hr exposure was 5.2 mg/L. In the subacute and subchronic studies, rats were exposed to air, 0.2-, 0.5-, or 1.5-mg/L fog oil smoke for 3.5 hr/day, 4 days/wk for either 4 or 13 wk. Both pulmonary and systemic effects were investigated one day after the exposure ceased. For one 13-wk exposure group, animals were also examined 4 wk after the exposure.

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Following the subacute 4-wk exposure to 1.5 mg/L, a multifocal pneumonitis was observed. Lung lavage had an elevated number of polymorphonuclear leukocytes (PMNs), alveolar macrophages, and total cells, and an increase in lavage fluid protein. Lung wet and dry weights were increased. A lesser increase in the number of alveolar macrophages and lung weights was observed after exposure to 0.5 mg/L. Pulmonary function tests revealed an increase in end expiratory volume (EEV) after 1.5 mg/L. Systemic effects after the subacute exposure were minimal. A decrease in zoxazolamine-induced paralysis time was observed following both 0.5 and 1.5 mg/L; however, no effect on pentobarbital-induced sleeping time was observed. Behavioral studies were negative, as were clinical chemistries and immune function tests. The subchronic 13-wk exposures resulted in decreased body weight and increased lung dry weights at both 0.5 and 1.5 mg/L. As in the 4-wk study, there was an increase in lavage fluid protein and EEV following the 1.5-mg/L concentration. Zoxazolamine-induced paralysis time, which was decreased after exposure to 0.2, 0.5, and 1.5 mg/L, correlated with an increase in aryl hydroxylhydrolase activity in the liver at all concentrations. There were no observable changes in immunology parameters or clinical chemistries. In general, males and females responded in a similar fashion. In conclusion, it appears that inhalation of fog oil smoke caused pulmonary effects indicative of an inflammatory response (a concentration-related increase in PMNs) a progressive granulomatous lesion at 1.5 mg/L. Systemically, there was an induction of cytochrome P_1-450, which could have significant implications for xenobiotic metabolism.

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NOTICE

Disclaimer

This paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

EXECUTIVE SUMMARY

Male and female, Sprague-Dawley, 60-day-old rats were exposed by inhalation to fog oil smoke (MIL SPEC. SGF-2), a light lubricating oil. Mortality studies showed fog oil smoke to be 100% lethal at 11.0 mg/L, 95% lethal at 5.0 mg/L, 20% lethal at 1.0 mg/L, and 0% lethal at 0.1 mg/L after a 6-hr exposure. The observed LC_{50} of fog oil smoke after a 3.5-hr exposure was 5.2 mg/L. Two exposure regimens were utilized; one, a 4-wk, subacute range-finding study, and two, a 13-wk subchronic study. During the range-finding study, various exposure concentrations, frequencies, and durations were investigated to determine the conditions to be used and parameters to be tested in the subchronic study. The 4-wk subacute exposure regimen (Phase II) consisted of two exposure concentrations (0.5 and 1.5 mg/L), two exposure frequencies (70 min and 3.5 hr/day) and two exposure durations (2 and 4 days/wk); both sexes were exposed and examined for specified parameters. A separate study of xenobiotic metabolism was also conducted to determine if the effects observed on hepatic metabolism were due solely to inhalation or if ingestion of the oil during preening was also a contributing factor. The subchronic exposure regimen consisted of the same two exposure concentrations (0.5 and 1.5 mg/L) for 3.5 hr/day, 4 days/wk for 13 wk, exposing males only (Phase III Part A). A separate group of animals was held for 4 wk after the 13-wk exposure terminated to determine if effects might be reversible, latent, or progressive. Control animals were subjected to similar exposure conditions but breathed filtered air. The average mass median aerodynamic diameter (MMAD) of the aerosol was 1 to 1.3 μm with a geometric standard deviation of 1.5.

Two additional subchronic studies were conducted. In one study, fog oil smoke concentrations of 0.2 and 0.5 mg/L were used with the same study regimen to determine a no-effect level (Phase III Part B). The other compared the effects of a 3.5-hr/day, 4-day/wk exposure for 13 wk at 1.5 mg/L in male and female rats (Phase III Part C).

Numerous biological parameters were investigated to determine the health effects of exposure to the fog oil smoke. The pulmonary parameters included histopathology, pulmonary function, cardiovascular physiology, pulmonary edema, and pulmonary cell differentials. Systemic parameters included histopathology, behavioral response, clinical chemistry, hematology, immunology, and xenobiotic metabolism.

Several effects were observed after 4 wk of exposure. A significant increase in lung wet weight and lavage fluid protein suggested the presence of edema in the lungs of animals exposed to 1.5-mg/L fog oil smoke for 4 wk. The increase observed in lung dry weights after 4 wk may be explained by the hypercellularity (i.e., influx of macrophages and polymorphonuclear leukocytes) observed in these same animals. These phagocytic cells are known to play an important role in the inflammatory responses of the lungs. Postmortem analysis of these animals also revealed a multifocal pneumonitis in rats exposed to the high concentration for 4 wk. Lung volumes, lung compliance, and ventilation distribution were not affected by fog oil smoke

exposure; however, end expiratory volume (EEV) increased, indicating an inflammatory response. We suspect that the increased EEV allowed the rats to ventilate the lung more efficiently with each breath, preventing a decrease in diffusing capacity and maintaining gas-exchange homeostasis. Of the parameters investigated that relate to xenobiotic metabolism, only zoxazolamine-induced paralysis time was significantly affected. This parameter, like pentobarbitol-induced sleeping time, is related to metabolism by the cytochrome P450 system, and thus the concentration-related decrease of paralysis time may indicate an induction of a specific isoenzyme of the hepatic cytochrome P450 system, an effect that is consistent with the polycyclic hydrocarbon content of the fog oil smoke.

Immediately following the 13-wk exposures, we observed effects similar to those following the 4-wk exposure. A significant increase in lung dry weight at both 0.5- and 1.5-mg/L fog oil smoke correlated with a significant infiltration of alveolar macrophages and polymorphonuclear leukocytes observed at these concentrations. Additional pathological findings included focal hemorrhage at 1.5 mg/L and peribronchial lymphoid hyperplasia with multiple pockets of macrophage accumulation in the peribronchial lymph nodes at both concentrations. As in the 4-wk study, zoxazolamine-induced paralysis time was significantly decreased at both concentrations. In addition, aryl hydrocarbon hydroxylase (AHH) activity significantly increased as fog oil smoke concentration increased. Because the fog oil smoke did not cause any changes in pentobarbital-induced sleeping time or liver cytochrome P450 levels, the correlation between AHH activity and paralysis time further substantiates the hypothesis that the constituents of the fog oil smoke induced the hepatic cytochrome P450 system, specifically P_1-450. This induction could have implications for drug therapy and metabolism of other foreign compounds by the liver.

Animals examined after a 4-wk recovery period also showed effects. A significant increase in lung wet and dry weights was observed after the 1.5-mg/L exposure. Histopathologically, animals exposed to 0.5 and 1.5 mg/L retained the accumulation of alveolar macrophages and exhibited hyperplasia in peribronchial lymph nodes. In addition, 3 of 10 male animals exposed to 1.5 mg/L exhibited multifocal granulomatous pneumonia. This development of granulomas after the cessation of exposure suggests a progressive lesion.

In Phase III Part B, an attempt was made to find a no-observable-effect level. However, even at 0.2 mg/L fog oil smoke, there was still an observed increase in alveolar macrophages in the lung, an increase in lavage fluid protein, an increase in AHH activity, and a decrease in zoxazolamine-induced paralysis time. These changes were concentration related.

In Phase III Part C, the response of male rats was compared to the response of female rats after exposure to 1.5 mg/L fog oil smoke. The response of both sexes was similar in direction (increase or decrease) but not necessarily similar in magnitude. However, when all the parameters assessed were evaluated, neither gender appeared to be more sensitive than the other.

In conclusion, 4-wk subacute and 13-wk subchronic inhalation exposures to fog oil smoke appear to cause an inflammatory response in the lungs of adult male and female rats, yet pulmonary function and gas exchange are not compromised. However, formation of granulomas appears to be progressive after cessation of exposure. Significant systemic effects were evident in the alteration of hepatic zoxazolamine-induced paralysis time and AHH activity, suggesting a compromised xenobiotic metabolism system.

FOREWORD

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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INTRODUCTION

Chemical smokes/obscurants are used by the military to conceal personnel, materiel, or installations from direct visual observation. The smoke from a petroleum distillate product is generated by injecting a light lubricating oil (SGF-2) into a heated engine exhaust manifold where it vaporizes and eventually recondenses in the atmosphere. Army personnel may be exposed to this chemical smoke when it is released into the environment in training or combat operations. Because this petroleum smoke is a large area screening obscurant, the duration of exposure is usually hours within a single day, and exposures may be repeated over consecutive days. Evaluation of the potential hazards posed by this smoke to human health is a necessary portion of the data base required to establish comprehensive health criteria for the field use of smokes and obscurants.

METHODS

FACILITY DESCRIPTION

A detailed description of the exposure facility has been previously published (Inhalation Toxicology of Fog Oil Obscurant Phase I: Inhalation Exposure Facility DTIC AD No. A144875). The exposure facility consisted of a 600-ft^2 exposure facility, a 160-ft^2 pre-exposure animal room (700 rat capacity) and a 200-ft^2 postexposure animal holding room (900 rat capacity). Six 27- x 27-in. stainless steel Rochester-type exposure chambers were installed in the exposure facility. Chamber air was drawn from room air filtered through chemical, bacteriological, and radiological filters.

The fog oil smoke generation and exhaust systems were based on the design developed at Oak Ridge National Laboratories (ORNL) under Interagency Agreement DOE No. 40-1016-70 with the modifications mentioned below. Each exposure chamber had its own generation and exhaust filtration system to allow maximum flexibility of chamber control and minimal disruption of exposure schedules.

The exposure facility provided automatic or manual (local servo) control of the fog oil smoke concentration in the exposure chamber. Automatic control significantly reduced operator time, increased exposure stability and provided accurate and rapid data processing. Real-time aerosol monitors (GCA/Environmental Instruments Model RAM-1, Bedford, MA) provided real-time concentration monitoring as well as the signal for automatic control. The RAM-1s were calibrated for the aerosol exposure by comparing the RAM-1 values to gravimetrically analyzed filter samples, thus converting the RAM-1 values to a mass concentration. The calibration curve enabled the computer to control the mass concentration in the chamber by varying the flow rate of the metering pump injecting the fog oil smoke into the Vycor-clad immersion heater. Filter samples were obtained and analyzed gravimetrically to determine the absolute concentration at intervals.

Tests were conducted to verify the stability of the aerosol generation and delivery system and the homogeneity of aerosol distribution within the chambers.

A study was conducted to determine the feasibility of applying an analytical chemical method for the routine chemical characterization of the SGF-2, lubricating oil and fog oil smoke. The major conclusion of the study was that the method proposed for chemical characterization of the fog oil using high performance liquid chromatography (HPLC) separations with subsequent gas chromatography analysis yielded little useful information and was extremely time consuming. Therefore, it was not feasible to use this technique for routine chemical characterization of the fog oils being used in these inhalation studies.

An alternative technique using an HPLC separation technique with monitoring at 254 nm was recommended. Separations of fog oil by HPLC techniques are reproducible for aromatic and semipolar fractions. By selecting 10 points on the absorbance curves, quantitative monitoring of the chemical nature of the fog oil, aerosol, and vapors could be accomplished. It was decided that EPA would take duplicate filter samples and ship one set to ORNL for analysis and characterization. USEPA is, therefore, not responsible for this data.

EXPOSURE CONDITIONS

All exposures were conducted according to methods described report DTIC AD No. A144875. Two quality control filter samples per exposure, per chamber per day were collected and weighed. The first sample was used to verify the accuracy of electronic concentration monitors. The second sample was archived for subsequent chemical analysis.

Phase I - Subacute Mortality Studies

Preliminary Tests

Eighty CD rats (Charles River Breeding Laboratories, Kingston, NY), 4 groups of 10 males and 10 females per group, were exposed for 6.0 hr to 11.0, 5.0, 1.0, or 0.1 mg/L of fog oil smoke, respectively. Fifty rats, five groups of five male and five female rats per group, were also exposed to 6.1, 3.4, or 2.0 mg/L for 4.0 hr; 5.3 mg/L for 3.5 hr; or 3.2 mg/L for 2.0 hr, respectively. In addition, 10 female rats were exposed to 9.8 mg/L for 2.0 hr.

Lethal Concentration-Median (LC_{50}) Studies

The concentrations chosen to be administered for each exposure time appear in Table 1. The selection of concentrations at 6.0 hr was based on the preliminary mortality study data. Because it was our understanding that the concentrations for each exposure period should attempt to bracket the mortality experience in the 10-50% range, the upper 99% confidence limit for the 6.0-hr exposure, or 2.84 mg/L, was chosen as the highest concentration to

TABLE 1. EXPOSURE CONCENTRATIONS FOR THE MORTALITY STUDIES

Exposure Time (hr)	Concentration (mg/L) Targeted	Actual
2.0	0.54	0.54
	3.80	3.90
	5.26	5.30
	7.27	7.20
	7.27	7.64
	8.00	8.02
	11.00	11.54
3.5	0.34	0.33
	2.37	2.43
	4.54	4.28
	4.54	4.63
	6.00	5.92
	8.70	8.88
6.0	0.77	0.80
	1.07	1.13
	1.48	1.46
	2.05	2.00
	2.84	2.82

be administered for the 6.0-hr exposure period. The lower concentration limit for the 6.0-hr duration was chosen as 0.77 mg/L. The maximum and minimum concentrations for the 2-hr exposure period were based on relative potency estimates obtained from the ORNL data. Probit analysis on the ORNL data indicated that a model with a common slope but different intercepts was reasonable. The relative potency based on this model for 6.0 hr, as compared to 2.0 hr for the effective concentration-median (EC_{50}), was approximately 2.56. Thus, the highest concentration selected for 2.0 hr was 2.56 x 2.84 = 7.27 mg/L. The lowest concentration selected for 2.0 hr was 2.56 x 0.21 = 0.54 mg/L. Due to low mortality rates of animals observed within 2 days after the first exposures at 2.0 and 3.5 hr, subsequent concentrations for these exposure durations were increased substantially over those originally proposed to ensure that we at least bracketed the EC_{50} for these times. Two-hour concentrations ranged from 0.54 to 11.54 mg/L; 3.5-hr exposures ranged from 0.33 to 8.88 mg/L. Also, the lowest targeted concentration for the 6.0-hr exposure was raised from 0.21 to 0.77 mg/L, given the low mortality at the other two exposure times on the first two exposure days. Thus, the targeted concentrations at 6.0 hr were logarithmically spaced between 0.77 mg/L and the originally proposed 2.84 mg/L.

Phase II - Subacute Range-Finding Studies

Animals

Male CD rats (Charles River Breeding Laboratories, Kingston, NY) were used throughout Phases II and III of this study except for immunology parameters that required inbred (Fischer 344) rats (same sex and source). Rats were received at approximately 60 days of age, and quarantined for at least 4 days. At the time exposures began, animals were 64-72 days old and weighed approximately 300 g.

Experimental Design - Study A

A completely randomized design included three factors, fog oil smoke concentration, exposure duration, and weekly frequency of exposure, in a 3 x 2 x 2 arrangement. The following definitions apply: concentration levels (C_1 = 1.5 mg/L, C_2 = 0.5 mg/L, A = Air); exposure duration (T_1 = 3.5 hr/day, T_2 = 70 min/day); weekly frequency (F_1 = 4 consecutive days/wk, F_2 = 2 consecutive days/wk. C_1 and C_2 were chosen because 0.5 mg/L was the lowest exposure concentration that could be achieved without making time-consuming alterations in the exposure facility, and 1.5 mg/L was the upper limit without risk of mortality and large weight differences between control and exposed animals. T_1 and T_2 were chosen because 3.5 hr/day was the longest time period without risk of mortality at 1.5 mg/L and 70 min is a log spacing below 2.0 hr. The weekly frequencies were chosen to simulate possible troop exposures. The total number of rats needed per treatment group was less for parameters for which more animals could be handled in a single day. The exposure regimen allowed for at least N-12 degrees of freedom with which to estimate experimental error when N is the total number of animals used to investigate a particular end point.

Experimental Design - Study B

Study B was designed to determine the contribution of oral exposure to fog oil smoke received by preening following the whole-body inhalation exposure. A 2 x 2 factorial arrangement of a completely randomized design was used. Filtered air and 1.5 mg/L fog oil smoke were administered to animals both in the nose-only and whole-body modes.

Phase III - Subchronic Study

Experimental Design

Part A

For each of two exposure concentration levels (0.5 and 1.5 mg/L) and filtered air, 80 male rats were exposed 3.5 hr/day, 4 days/wk for 13 wk. Half of the animals in each exposure group were used to evaluate biological parameters on the day after the last exposure. The remaining half were evaluated 4 wk after the last exposure to assess recovery.

Part B

For each exposure concentration (0.2 and 0.5 mg/L) and filtered air, 40 male rats were exposed 3.5 hr/day, 4 days/wk, for 13 wk. Selected biological parameters were evaluated on the day after the last exposure.

Part C

Forty male and forty female rats were exposed to filtered air and 1.5 mg/L fog oil smoke, 3.5 hr/day, for 13 wk. Selected biological parameters were evaluated on the day after the last exposure.

STATISTICAL ANALYSIS

LC_{50} Studies

Probit analysis (Finney, 1971) and logit analysis were used to analyze binary response data generated from data tested at various concentration levels. The probability of a subject responding at concentration X is described as

$$P = \phi(\beta_0 + \beta_1 X) \quad (1)$$

where

ϕ = unit normal or logistic cumulative distribution function,
X = concentration or log concentration,
β_0 = the intercept, and
β_1 = the slope of the probit or logit regression line.

The analysis of probits or logits involving linear combinations of more than one independent variable means that Equation 1 can be extended to

$$P = \phi\left(\beta_0 + \sum_{i=1}^{n} \beta_i X_i\right) \quad (2)$$

where the X_i's may represent continuous or classification (dummy) variables or interactions between variables. Thus, the model given by Equation 2 was used within either an analysis of variance (ANOVA) or a multiple regression framework. Tests of the various model terms were performed by examining chi-square (χ^2) statistics derived from likelihood ratios estimated by fitting reduced models. Model parameters were estimated using maximum likelihood techniques.

Phase II and Phase III Studies

For end points in which more than one biological parameter was measured, both univariate and multivariate ANOVA models were fit to the data. Preliminary analysis of each end point variable examined homogeneity of variance among the various treatment groups and the normality of ANOVA model residuals. In the event that either of the above ANOVA assumptions was strongly violated, analysis of a mathematically transformed variable or analysis by nonparametric methods was considered. When there was no evidence of an interaction between the two factors of interest (i.e., fog oil smoke concentration and either exposure duration, time, or sex), main effects were examined for statistical significance. In addition, for each variable, a series of contrasts testing the control (air) group mean versus each fog oil smoke concentration mean at each level of the second factor (time, duration, or sex) was used. Significant interactions were explored through post hoc examination of standardized differences of least square means involved (subtests, t tests, and post hoc t tests refer to this).

One of the objectives of Phase III Part B (fog oil smoke exposures at 0.2 and 0.5 mg/L, and filtered air) was to add another concentration to the existing 13-wk data for these parameters and then to determine the lowest observable effect level. This involves pooling data from two studies. For end points whose study represented a replicate (REP) of the original study, it was first determined if the responses at the two concentrations (CONC) shared by animals in the first replicate (i.e., filtered air and 0.5 mg/L) were similar. This was accomplished by looking at a two-way ANOVA model with REP at two levels and CONC at two levels. If there appeared to be either a sizable REP by CONC interaction or a REP main effect, the data from the two groups were not pooled. A two-sided Williams' test was then applied to each replicate. If no effects involving REP were significant, then data from the two replicates were combined, and Williams' test was applied to the combined data. Only in the latter case were responses at 0.2 mg/L compared with those at 1.5 mg/L.

A two-way ANOVA model was fit to the data generated in the study comparing sexes. The model included terms to test for the interaction of gender and fog oil smoke concentrations, as well as their main effects. When multiple responses per animal were present, an analogous multivariate ANOVA model was fit to data to test for multidimensional effects prior to examining any univariate responses. Significance probabilities associated with each set of the above contrasts were adjusted so an overall Type 1 error rate of 5% was not exceeded. Multiple comparisons were adjusted for using the Bonferroni correction factor.

BIOLOGICAL PARAMETERS

Body Weight

All animals were weighed before the first exposure and after the last exposure. In addition, 10 animals per treatment group were selected at random from among those to be used for the biological parameters. These animals were weighed before the first exposure each week and after the last exposure each week.

Histopathology

Necropsies and histopathology were performed on each animal. Necropsy included the external examination of the carcass for gross lesions and the examination and fixation of the following tissues in 10% neutral buffered formalin except those indicated for which Bouin's fixative was used.

- Brain[a]
- Pituitary
- Eyes
- Spinal cord
- Salivary glands
- Mandibular lymph node
- Peripheral nerve (Sciatic)
- Urinary bladder
- Nasal cavity and turbinates
- Thyroid
- Parathyroid
- Thymus
- Trachea
- Esophagus
- Epididymides[b]
- Lung[a]
- Heart
- Aorta
- Ovaries
- Uterus
- Liver[a]
- Thigh muscle
- Spleen
- Prostate
- Adrenals
- Cecum
- Colon
- Pancreas
- Tongue
- Stomach
- Duodenum
- Jejunum
- Ileum
- Skin
- Testes[a,b]
- Mammary gland
- Sternebrae containing bone marrow
- Kidneys[a]
- Seminal vesicles
- Mesenteric lymph node

All tissues were examined in situ, dissected from the carcass, trimmed, weighed, and reexamined before fixation. Tissue samples for fixation did not exceed a thickness of 0.5 cm. Lungs were fixed in their entirety after being reinflated with formalin fixative under 25- to 30-cm water pressure. The calvarium was removed and the brain freed and weighed. Then the entire skull including the nasal cavity (turbinates) and pituitary gland was placed in fixative.

The following tissues were sectioned and stained with hematoxylin and eosin using routine histopathologic procedures:

- Gross lesions
- Duodenum
- Liver
- Testes/Epididymides
- Lungs (Two sections, left lobe and right diaphragmatic lobe)
- Nasal cavity and turbinates (Three separate sections)
- Heart
- Stomach
- Larynx
- Trachea (Distal)
- Kidneys
- Eyes
- Skin (Mid-dorsal region)
- Peribronchial lymph nodes

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[a] These organs were weighed.
[b] Bouin's fixative.

Pulmonary Physiology

The animals were anesthetized with sodium pentobarbital (50 mg/kg Nembutal), tracheostomized, and the following measurements performed: lung volumes, compliance (quasi-static pressure volume), diffusing capacity (DL_{co}), distribution of ventilation (N_2 washout), and lung wet and dry weights. These procedures are covered by "Pulmonary Function Evaluation in Small Laboratory Animals" and "Analysis of Respiratory Gas Samples by Gas Chromatography" (SOP JT-01-00).

Pulmonary Edema

Animals were anesthesized with Nembutal (50 mg/kg) and lungs were lavaged according to SOP GH-07-00, "Obtaining Lavage Fluid from Animals." Samples of lavage fluid were stored frozen (-20°C) until assayed for total protein content according to SOP GH-03-00, "Lowry Protein Assay for Lavage Fluid."

Pulmonary Cells

Animals were anesthetized with Nembutal (50 mg/kg), intratracheally injected with *Streptococcus* sp. Group C, and lungs were lavaged 30 min postinjection. Total and differential cell counts and cell viability were assayed according to SOP JI-03-01.

Cardiopulmonary Physiology

Rats were anesthetized with Nembutal (50 mg/kg) and surgically implanted with an intrapleural or a carotid arterial catheter following the last exposure. The next day the following were measured: lung weight, lung volume, blood pressure, electrocardiogram (ECG), and blood pH, pO_2, and pCO_2. Procedures for these measurements in rats are described in SOP JT-01-00. Blood gas analysis was done using a radiometer (BMS 3 MK 2) as specified in the user manual.

Behavioral Response

Procedures for determining rat activity in a figure-eight maze are detailed in the SOP entitled "Figure-Eight Mazes." The activity of rats in the maze was recorded via eight photodiode pairs mounted in the maze.

Clinical Chemistry

Following exposure, blood samples were collected from the dorsal aorta immediately after cervical dislocation. To evaluate potential target organ toxicity, the following clinical chemistry assays were performed according to procedures contained in the Baker Instruments Centrifichem 400 Operators Manual (SOPs RJ-07-00 through RJ-40-00):

Albumin
Aldolase
Alkaline phosphatase
Amylase

Bilirubin (Total)
Blood urea nitrogen
Calcium
Cholinesterase
Cholesterol
Creatine kinase
Creatinine
Glucose
Lactate dehydrogenase (LDH)
Leucine aminopeptidase
Phosphate (Inorganic)
Protein (Total)
Aspartate aminotransferase (SGOT)
Alanine aminotransferase (SGPT)
Triglyceride
Uric acid

The lactate dehydrogenase and triglyceride assays were performed on the same day the blood samples were collected. The additional serum samples were stored at -80°C, and the remaining analyses were completed the following workday.

Hematology

Animals were anesthetized with Nembutal (50 mg/kg) and bled from the aorta. Complete blood count and leukocyte differential tests were performed.

Immunology

Fischer 344 rats (for the 4-wk exposures) and CD rats (for the 13-wk exposures) were anesthetized with Nembutal (50 mg/kg) and bled from the aorta, and spleens were removed. Spleens and peripheral blood from individual rats were processed, and the response of cells to phytohemagglutinin (PHA), Concanavalin A (Con A), and pokeweed mitogen (PWM) were assessed according to SOP MS-13-00, "Standard Procedures for Mitogen Responsiveness Test for Rats." The natural killer (NK) cell activity of the same spleen cell preparations was assessed using SOP MS-14-00, "Natural Killer Cell (NK Cell) Assay for Rats."

Xenobiotic Metabolism

Pentobarbital-induced sleeping time, cytochrome P450 levels, aryl hydrocarbon hydroxylase activity (AHH), and zoxazolamine-induced paralysis time were investigated to determine whether the fog oil smoke affected xenobiotic metabolism. Rats were given intramuscular injections of 25 mg/kg Nembutal. Time to loss of righting reflex and sleeping time were assayed according to SOP JI-02-00. Animals in a separate group were injected with 100 mg/kg zoxazolamine and the induction time and paralysis time were assayed according to SOP JI-02-01. All studies were routinely conducted in an isolated temperature- (24° ± 1°C) and humidity- (46 ± 2%) controlled room to minimize the influence of temperature on the results.

A sample of the liver microsomal fraction was obtained according to SOP EG-02-00. The sample was then divided into two aliquots, one for the cytochrome P450 assay and one for the AHH activity assay. Cytochrome P450 levels were determined according to the methods described in detail in SOP EG-05-00. Microsomes were diluted with 1M K_2HPO_4/1M KH_2PO_4 (pH 7.0) buffer and the carbon monoxide-difference spectra of sodium dithionate-reduced microsomes determined in duplicate on a Cary spectrophotometer using an extinction coefficient of 91 $cm^{-1}mM^{-1}$. The AHH assay was done according to the methods detailed in the SOP entitled "Radioactive Assay of Benzo(a)pyrene (BAP) Monoxygenase (aryl hydrocarbon hydroxylase)." The microsomal sample was mixed with purified tritiated BAP and other substrates, and incubated 10-30 min at 37°C. The reaction was stopped, the sample extracted with hexane, and aliquots from the upper and lower phases were counted in a liquid scintillation counter to determine the relative amounts of metabolized and unmetabolized BAP. Appropriate calculations were done to determine the total moles of BAP metabolized per milligram of microsomal protein.

All SOPs are available to the Project Officer or his representative from the USEPA Toxicology Branch upon request. A summary of the biological parameters measured in Phase II and III appears in Table 2.

RESULTS

ENGINEERING

Distribution Studies

Aerosol distribution studies were conducted to determine the distribution of the smoke in each chamber (DTIC AD No. A144875).

In summary, when aerosol was generated in the chambers without animals, the estimate of the covariate was found to be statistically different from one. In addition, there were main effect differences from front to back and from right to left. The maximal difference from any one of these effects was 2.4%, which was well within engineering feasibility constraints. Furthermore, when the study was repeated with animals using similar concentrations, none of these differences were found. The test for the covariate's equality to one could be rejected. Among the factors, one interaction effect appeared to have statistical significance, but differences among the means could not be detected when subtesting was performed.

Chemical Characterization

Using HPLC, individual chromatograms were obtained for 37 samples from the four fog oil smoke exposure chambers. A typical chromatogram is shown in Figure 1. In each chromatogram, the peak heights of 12 major peaks were summed. The individual peak heights were then divided by the total of all the peak heights yielding a normalized peak height expressed in percentages for each chromatogram. Mean peak heights were then obtained for each exposure chamber (Figure 2). The results indicated that there was a general homogeneity (within 2%) for the chromatograms of the different chambers. The greatest difference between chambers occurred in peaks K and L. The difference may be due to the difference in fog oil smoke concentrations (i.e., 1.5 vs. 0.5 mg/L). Because K and L were minor peaks and the difference was small, we did not consider this to be a problem.

TABLE 2. BIOLOGICAL PARAMETERS MEASURED IN FOG OIL SMOKE STUDIES

Parameter	Phase II	Phase III		
		Part A	Part B	Part C
AHH activity	X	X	X	X
Behavior (Fig. 8 maze)	X			
Body weight	X	X	X	X
Cardiopulmonary	X			
Clinical chemistry	X	X		
Cytochrome P450	X	X	X	X
Edema	X[a]	X	X	X
Hematology	X	X	X	X
Immunology	X	X		
Pathology	X[a]	X	X	X
Pentobarbital-induced sleeping time	X			
Pulmonary cells	X		X	X
Pulmonary physiology	X	X	X	X
Zoxazolamine-induced paralysis time	X	X	X	X

a. Females and males.

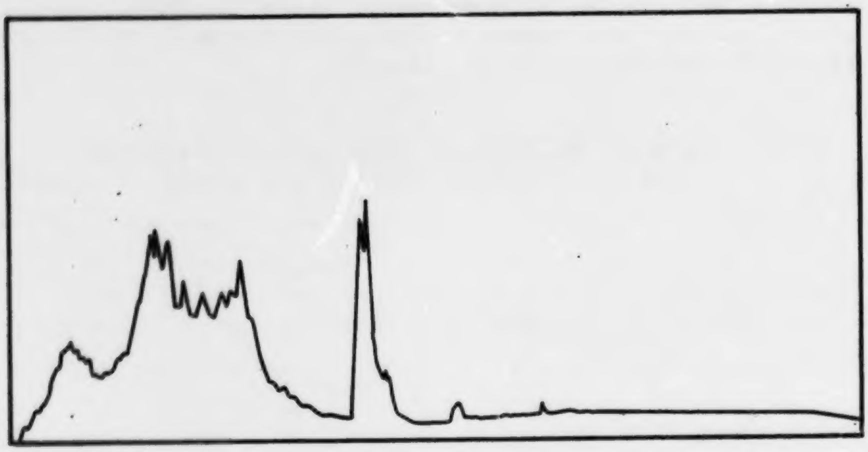

Figure 1. Typical HPLC chromatogram of fog oil sample.

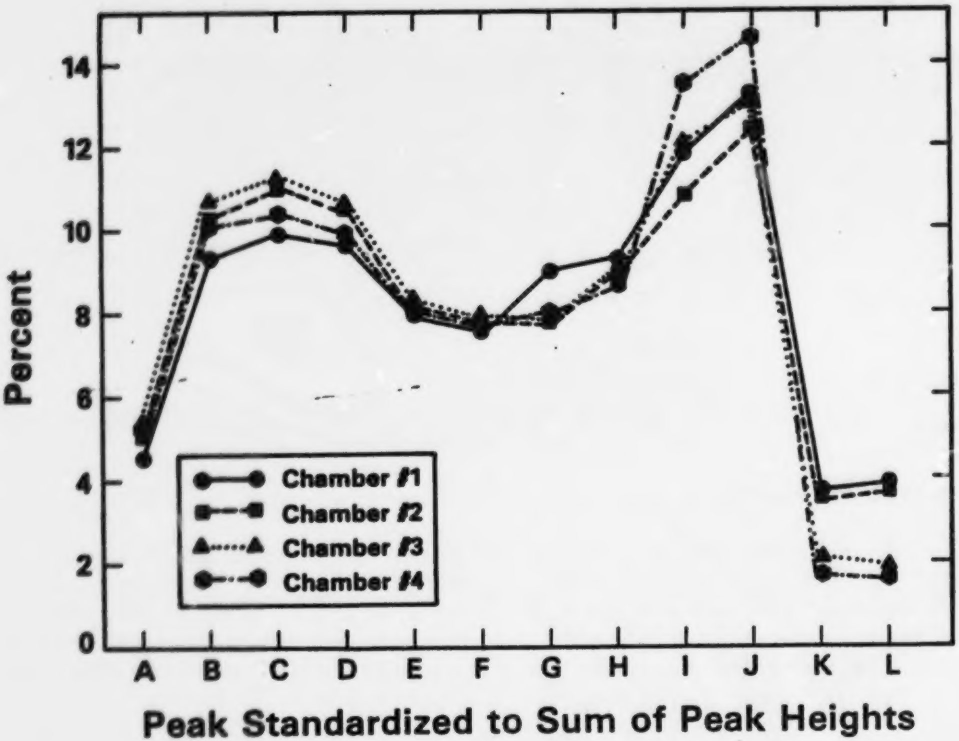

Figure 2. Comparison of the chemical homogeneity of the 4 fog oil smoke chambers during the 4-wk exposures.

Particle Size Distribution and Exposure Concentration

Particle size was determined for all four chambers during each phase of the study. The mass median aerodynamic diameter (MMAD), geometric standard deviation, and the mean exposure concentrations with the standard deviations for Phases II and III appear in Table 3.

TABLE 3. PARTICLE SIZING AND EXPOSURE CONCENTRATIONS PER FOG OIL SMOKE CHAMBER FOR PHASES II AND III

Phase	Chamber	MMAD	Geometric Standard Deviation	Target Conc.(mg/L)	Actual Conc.(mg/L)
II	1	1.26 ± 0.05[a]	1.46	0.5	0.50 ± .01
	2	1.04 ± 0.24	1.52	0.5	0.49 ± .01
	3	1.02 ± 0.24	1.52	1.5	1.49 ± .03
	4	1.16 ± 0.28	1.49	1.5	1.51 ± .04
IIIA	1	1.18 ± 0.04	1.46	0.50	0.50 ± 0.02
	2	1.19 ± 0.07	1.48	0.50	0.51 ± 0.02
	3	1.35 ± 0.06	1.46	1.50	1.50 ± 0.03
	4	1.32 ± 0.10	1.45	1.50	1.50 ± 0.02
IIIB	1	0.85 ± 0.07	1.66	0.20	0.20 ± 0.01
	2	1.08 ± 0.15	1.54	0.50	0.50 ± 0.01
IIIC	3	1.11 ± 0.06	1.49	1.50	1.50 ± 0.04
	4	1.19 ± 0.08	1.47	1.50	1.50 ± 0.05

a. Mean ± standard deviation.

BIOLOGY

Phase I

Mortality Studies

Four groups of 10 male and 10 female rats each were exposed to 11.0, 5.0, 1.0, or 0.1 mg/L of the fog oil smoke for 6.0 hr. Mortality was observed for 14 days postexposure. Mortality observed at 11.0 mg/L was 100%, 95% at 5.0 mg/L, 20% at 1.0 mg/L, and 0% at 0.1 mg/L. Gross observation of tissues from dead animals indicated severely hemorrhagic lungs and bleeding from the nares prior to death.

Prior to performing the LC_{50} studies, several preliminary studies were completed so that a relevant estimate of concentration could be determined. Ten female rats were exposed to 9.78 ± 0.76 (SD) mg/L for 2.0 hr. Groups of five male and five female rats each were exposed for 4.0 hr to 1.95 ± 0.42 mg/L, 3.40 ± 0.33 mg/L, or 6.18 ± 0.67 mg/L. Fifteen female rats were exposed to 1.01 ± 0.03 mg/L for 6.0 hr, and five male and five female rats were exposed to 5.28 ± 0.23 mg/L for 3.5 hr or 3.18 ± 0.10 mg/L for 2.0 hr.

LC_{50} Studies

A total of 338 rats (160 males, 178 females) was exposed to various concentrations of fog oil smoke for either 2.0, 3.5, or 6.0 hr. The 2.0-hr concentrations ranged from 0.54 to 11.54 mg/L; 3.5-hr exposures ranged from 0.33 to 8.88 mg/L, and 6.0-hr exposures ranged from 0.80 to 2.82 mg/L. The percentages of rats dying by sex, exposure time, and exposure concentration appear in Table 4. The observed mortality rates for each exposure duration, pooled over sex, are plotted in Figure 3. The rates for each length of exposure, for each sex, are displayed in Figure 4.

Using the UNIVAC version of Generalized Linear Interactive Modeling (GLIM), various multiple probit and logit regression models were applied to the study data. In the form of Equation 2 (see Methods Section), the largest models included variables for concentration, exposure time, and age, and all first- and second-order interactions of these variables. For one set of analyses, logarithms of all continuous variables were used; the other set simply used the arithmetic values.

The general strategy employed in the model-fitting process was to find the most parsimonious model with respect to model terms that explained a substantial amount of the variation in the rats' mortality rates. By successively dropping each interaction term from the model while retaining main effects and all other interactions of the same or lower order, chi-square statistics derived by comparing the likelihood from the enlarged model to the reduced model were obtained to test for the contribution of each model term. Tests of main effects were performed only if the effect was not involved in a significant ($\alpha = 0.05$) interaction.

Summaries of these tests for both probit and logit models, with and without using logarithms of continuous type variables, are shown in Table 5. If only effects that contribute significantly to form a probability model to characterize rat mortality are retained, the simplest model that could be fit to the data was a multiple logistic model containing an overall mean and terms for concentration, exposure time (TIME), and the interaction of concentration and exposure time. In the form of Equation 2, this model estimates the risk of a rat dying, under the conditions imposed in this experiment, as

TABLE 4. MORTALITY DATA FROM LC_{50} STUDIES

Time (hr)	Concentration Received (mg/L)	% Mortality	
		Male	Female
2.0	0.54	0	0
	3.90	20	40
	5.30	0	10
	7.20[a]	-	33
	7.64	10	20
	8.02	40	60
	11.54	100	80
3.5	0.33	0	0
	2.43	0	20
	4.28	10	20
	4.63[a]	-	22
	5.92	80	90
	8.88	90	100
6.0	0.80	0	0
	1.13	0	0
	1.46	0	10
	2.00	0	0
	2.82	60	60

a. These additional tests were done because a failure in the watering system caused some of the animals in the 7.64-mg/L-2.0-hr group and the 4.28-mg/L-3.5-hr group to be without water for a period of time prior to exposure. This incident did not appear to affect the percentage of mortality observed.

$$P = \phi\,[-4.41 - (0.131 \times \text{CONC}) - (0.265 \times \text{TIME}) + (0.350 \times \text{CONC} \times \text{TIME})], \quad (3)$$

where ϕ refers to the cumulative logistic function:

$$\phi\,(z) = 1/[1 + e^{-z}]. \quad (4)$$

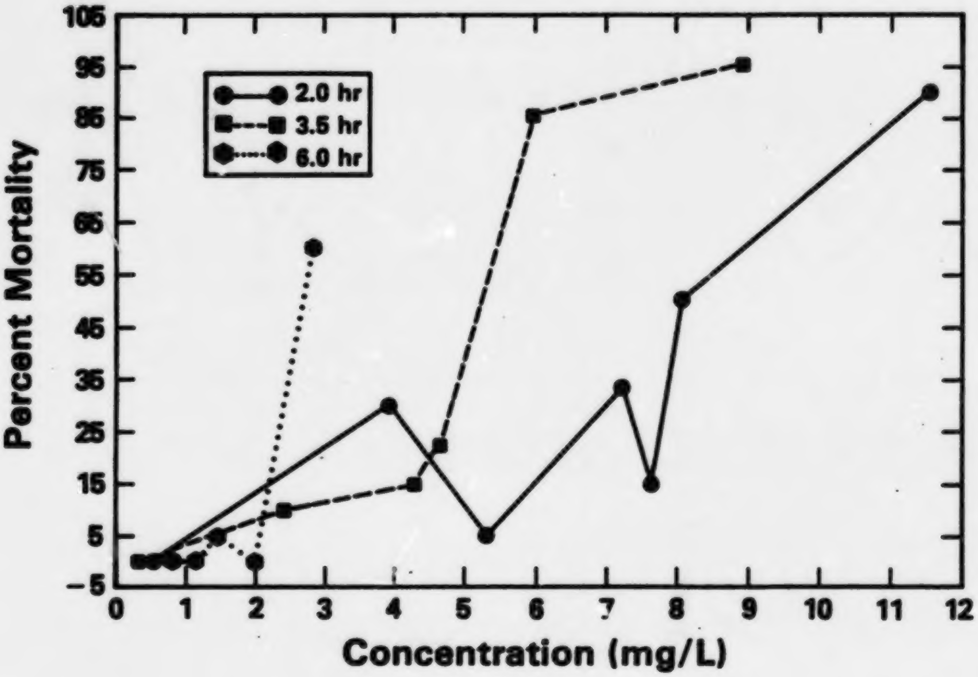

Figure 3. Observed death rates pooled over sex for the acute mortality study.

If the specific exposure times used in this study are substituted for TIME in Equation 3, Equation 4 can be written in the form of Equation 3. For example, for the 2.0-hr exposure, Equation 3 translates to

$$P = \phi\ [-4.94 + (0.569 \times \text{CONC})].$$

Similarly, for 3.5 hr

$$P = \phi\ [-5.34 + (1.09 \times \text{CONC})],$$

and for 6.0 hr

$$P = \phi\ [-6.00 + (1.97 \times \text{CONC})].$$

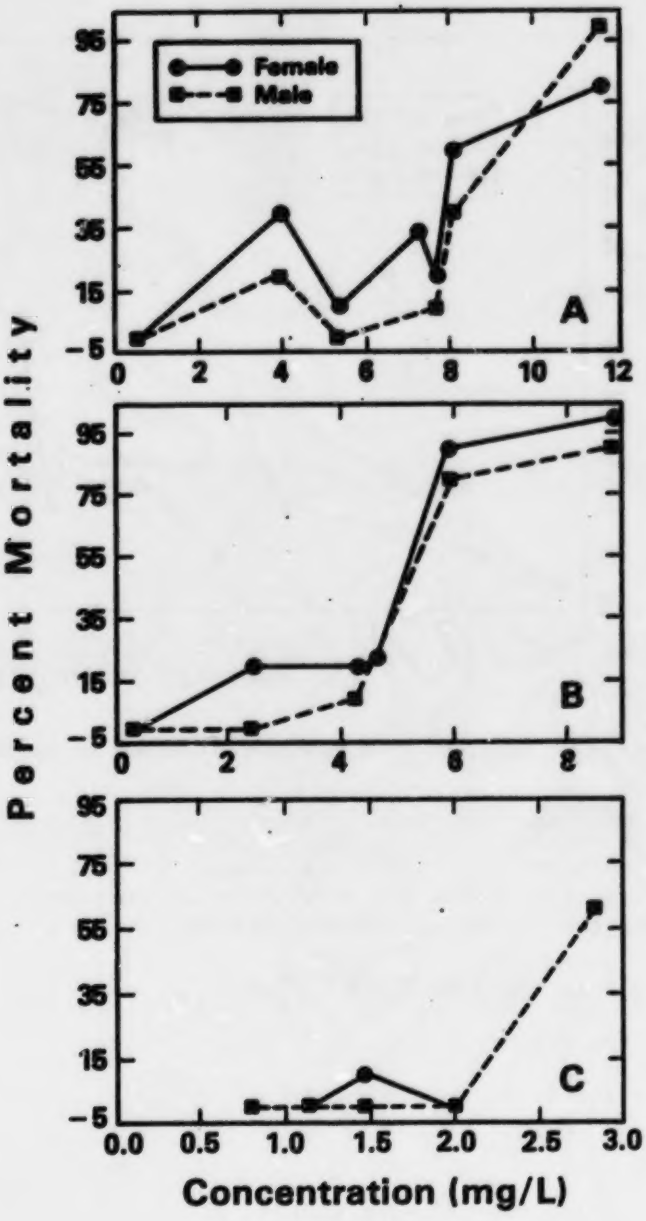

Figure 4. Observed deaths by gender after fog oil smoke exposure. A. 2.0-hr exposure; B. 3.5-hr exposure; C. 6.0-hr exposure.

TABLE 5. LIKELIHOOD RATIO TESTS[a] OF CONTRIBUTIONS OF EFFECTS USING MULTIPLE PROBIT AND LOGIT MODELS

		Model			
		Probit		Logit	
Effect	Degrees of Freedom	Logs χ^2	No. Logs χ^2	Logs χ^2	No. Logs χ^2
Residual	323	228.7	225.7	227.1	224.3
Dose	1	NT[b]	NT	NT	NT
Time	1	NT	NT	41.7**	NT
Sex	1	2.1	2.5	NT	2.0
Age	1	NT	0.2	NT	0.1
Dose x time	1	0.0	21.2*	3.4	21.5**
Dose x sex	1	3.3	1.5	3.9*	2.5
Dose x age	1	5.3*	3.9*	5.0*	3.1
Time x sex	1	1.1	0.3	1.8	1.1
Time x age	1	3.9*	1.5	3.2	1.0
Sex x age	1	2.0	0.8	2.0	0.5
Dose x time x sex	1	0.1	0.0	0.2	0.0
Dose x time x age	1	0.5	1.3	0.4	1.3
Time x sex x age	1	3.8	0.9	2.9	0.6
Dose x sex x age	1	2.6	1.1	2.3	0.8

a. From GLIM.

b. Not tested.

* $p(\chi^2 > \text{value}) < 0.05$.

** $p(\chi^2 > \text{value}) < 0.005$.

The fit of Equation 3 to the observed mortality rates is shown in Figure 5. Estimates of the concentrations resulting in various mortality percentiles could be estimated from Equation 3 for each exposure time. Also, by estimating asymptotic variances for each of these concentrations, appropriate confidence limits could also be calculated. However, given the large amount of heterogeneity of variance of observed deaths about the logistic regression lines, confidence limits estimated by such an approach might be too narrow.

Therefore, separate logit models in the form of Equation 1 were fit to data for each exposure time using the Probit and Logit (PROLO) program (Russell et al., 1977). The 2.0-hr data suffered from heterogeneity of variance. The 6.0-hr data reflected too high an index of significance for potency estimation. Only the data corresponding to 3.5 hr were sufficient enough to use for the estimation of fiducial limits. Estimated concentrations corresponding to the 10-50% mortality rates at 3.5 hr are shown in Table 6, as well as 90% fiducial limits for the true concentrations reflecting these rates. The risk of a rat dying from a single 3.5-hr exposure is estimated as

$$P = \phi\,(-5.75 + 1.11 \times \text{CONC}) \qquad (5)$$

where ϕ is defined as in Equation 4.

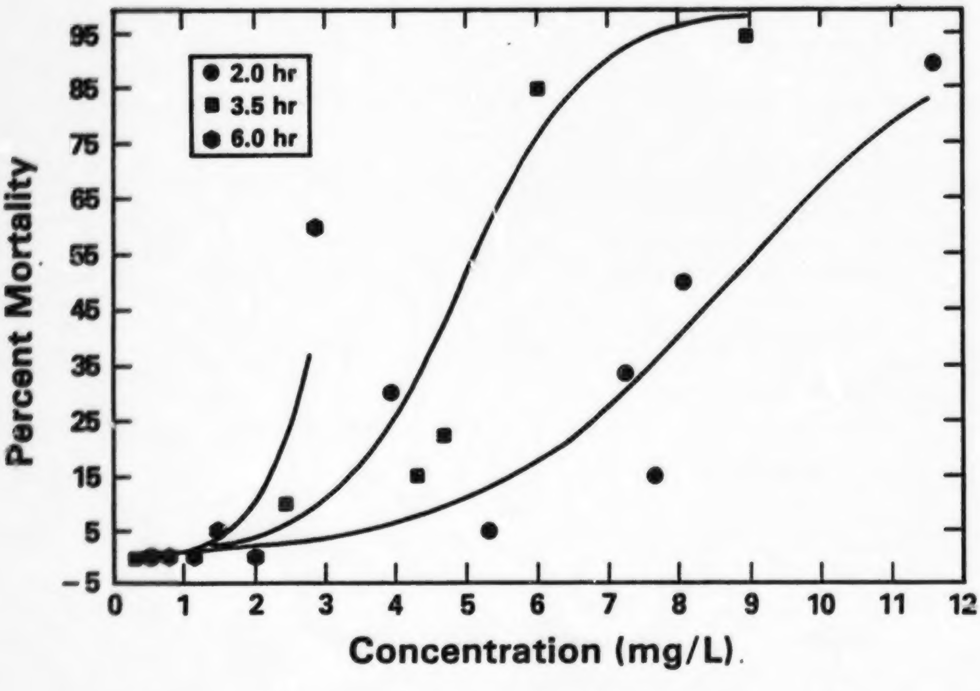

Figure 5. Fit of multiple logistic model (Equation 3) to mortality data.

TABLE 6. ESTIMATES OF CONCENTRATIONS CORRESPONDING TO 10 TO 50% MORTALITY AFTER 3.5-HR EXPOSURE TO FOG OIL SMOKE

% Mortality	Concentration (mg/L)	90% Fiducial Limits (mg/L)
10	3.21	(0.32, 4.11)
20	3.94	(2.03, 4.71)
30	4.42	(3.07, 5.21)
40	4.82	(3.81, 5.74)
50	5.19	(4.35, 6.35)

Histopathologic evaluation of samples taken during the subacute LC_{50} study was conducted. The mortality data from the LC_{50} study, with the addition of a summary of histopathologic observations for each group, are presented in Table 7. Tissues from dead animals or animals killed just prior to death (moribund killed) were taken from two animals per sex per exposure group. For some exposure groups, fewer than two animals per sex died, in which case histopathologic studies were conducted on fewer than four animals. In addition, two animals per sex from four exposure groups were terminally killed after a 2-wk postexposure period.

No significant histopathologic changes were observed in the upper respiratory tract (nasal cavity, larynx, and trachea) of rats that died, were moribund killed, or were terminally killed. A minimal to moderate number of histiocytic macrophages were present in the sinusoids of the peribronchial lymph nodes from rats that died, from moribund killed rats, and from rats that were terminally killed. The number of histiocytes was greater than expected in a lymph node from a normal rat; however, sinusoidal histiocytosis would be expected sequelae following the pulmonary injury described below.

The most severe pulmonary lesions were observed in the lungs of animals that died or were moribund killed. Histopathologic changes were less severe in rats killed after the 2-wk postexposure period. Lung changes in rats that died or were moribund killed were indicative of vascular injury. The connective tissue around most pulmonary vessels was distended by a minimal to moderate amount of edematous fluid, and various degrees of subacute inflammatory reaction (neutrophils and macrophages) were present around the vessels. The lumens of vessels were lined by inflammatory cells. Multiple focal hemorrhages, strands of fibrin, and scattered macrophages were present in the alveolar lumens. Eosinophilic edematous fluid was noted in the pulmonary alveoli. Several lungs contained eosinophilic and degenerate alveolar walls, and occasionally an alveolar wall was thickened. Some of the

TABLE 7. EFFECTS OF A SINGLE EXPOSURE TO FOG OIL SMOKE

Duration Time (hr)	Actual Concentration Received (mg/L)	% Mortality			Histopathology Summary on Dead and Moribund	
		M	F	M+F	Gross Observation of Lungs	Microscopic Observations
2.0	0.54	0	0	0		-
	3.90^a	20	40	30	Diffusely dark red	Focal hemorrhage
	5.30	0	10	5	Diffusely dark red	Focal hemorrhage
	7.20^b	-	33	-	-	-
	7.64	10	20	15	Diffusely dark red; bloody nostril in 1 male	Focal hemorrhage
	8.02^a	40	60	50	Diffusely dark red	Focal hemorrhage
	11.54	100	80	90	Diffusely dark red; blood around nose & mouth of 1 male	Focal hemorrhage
3.5	0.33	0	0	0	-	-
	2.43	0	20	10	Diffusely dark red	Focal hemorrhage
	4.28	10	20	15	Diffusely dark red or dark red	Focal hemorrhage
	4.63^b	-	-	-	-	-
	5.92	80	90	85	Diffusely dark red	Focal hemorrhage
	8.88	90	100	95	Diffusely dark or light red	Focal hemorrhage
6.0	0.80	0	0	0	-	-
	1.13	0	0	0	-	-
	1.46	0	10	5	Diffusely dark red	Focal hemorrhage
	2.00	0	0	0	-	-
	2.82^a	60	60	60	Diffusely dark or pale red	Focal hemorrhage

a. Groups from which additional animals were terminally killed 2 wk postexposure. At that time there were no gross lesions; microscopically slight, minimal, or no lesions were observed.

b. These additional tests were done because a failure in the watering system caused some of the animals in the 7.64-mg/L-2.0-hr group and in the 4.28-mg/L-3.5-hr group to be without water for a period of time prior to exposure. This incident did not appear to affect the percentage of mortality observed. Histopathology tissues were not taken from these groups.

thickened walls were due to the presence of inflammatory cells, and other thickened walls were due to the early proliferation of Type II epithelial cells.

After the 2-wk postexposure period, no histopathologic changes were present in the respiratory tract of several exposed rats. Minimal to slight degrees of residual lesions were noted in other terminally killed rats. The decreased severity of pulmonary lesions in these rats indicated that pulmonary repair was occurring.

Preliminary Mortality Studies for Phase II

In preparation for the 4-wk range-finding studies, animals were exposed at the concentrations (CONC) and times listed in Table 8 for 4 days/wk for 4 wk to determine a CONC x TIME combination that would not cause death. While essentially no mortalities occurred during these exposures (Table 8), another problem was identified. Animals exposed to fog oil smoke for 4 wk had substantially decreased weight gains compared to nonexposed animals. Animals appeared to be anorectic during the 4 days of exposure but did eat over the 3-day period of nonexposure. Water intake also decreased. Table 9 shows the weight changes observed in animals weighed prior to treatment and after treatment.

Because of weight differences between control and exposed animals, several additional studies were conducted. A histopathology study was undertaken to determine if lesions occurred in the digestive tract or liver. Such lesions could indicate if ingestion of the oil was a problem. It should be noted that the absence of such lesions would not necessarily mean ingestion was not a problem because changes, for example in liver function, could occur without producing lesions detectable by routine histopathology. Three male and three female rats were exposed to 2.0 mg/L, 3.5 hr/day, 4 days/wk for 4 wk. No significant treatment-related lesions were noted in samples taken from the digestive tract or liver. Lesions in the lung were similar to those previously noted.

Because the weight differences noted between control and exposed animals were discovered as a by-product of another experiment, an experiment designed specifically to study weight differences was performed to verify this observation. Four groups of rats each containing 10 males and 10 females were treated for 3.5 hr/day, 4 days/wk, for 4 wk as follows: control animals held in holding racks (Air-NC), animals in a control chamber treated exactly as animals that received a whole-body exposure of fog oil smoke (Air-WB), wholebody exposure to 0.3 mg/L fog oil smoke (0.3-WB), and whole-body exposure to 2.0 mg/L (2.0-WB). For the second through fourth weeks of exposure, two additional groups were added. Both of these groups were placed in Plexiglass modules (MD) to obtain a nose-only exposure, and thus prevent ingestion due to preening. One group was exposed to 2.0-mg/L fog oil smoke (2.0-MD), and one group was placed in the control chamber (Air-MD). Weights of animals were determined every Monday prior to beginning exposures for that week and every Thursday following exposures. Total weight gain for the 4-wk period was determined for the whole-body exposures as the animal's fourth week

TABLE 8. MORTALITY RESULTS OF CONCENTRATION x TIME STUDY

Total No. of Days Exposed	Average Concentration (mg/L)	Exposure Length (hr)	Dead/Living	Sex
8	0.31 ± 0.02[a]	3.5	0/10	F
8	0.31 ± 0.02	3.5	0/10	M
12	0.51 ± 0.03	3.5	0/10	F
12	0.51 ± 0.03	3.5	0/10	M
16	1.06 ± 0.08	3.5	0/10	F
16	1.06 ± 0.08	3.5	0/10	M
16	1.54 ± 0.04	3.5	0/10	F
16	1.54 ± 0.04	3.5	0/10	M
16	2.05 ± 0.05	3.5	1/10	F
16	2.05 ± 0.05	3.5	0/10	M
16	1.06 ± 0.05	6.0	1/10	F
16	1.06 ± 0.05	6.0	0/10	M

[a] Mean ± standard deviation.

TABLE 9. EFFECT OF FOG OIL SMOKE EXPOSURE ON ANIMAL WEIGHT

Exposure Regimen[a]	Weight Change Over a 4-wk Exposure Period	
	Male	Female
Nonexposed	+191.8	+74.3
2.0 mg/L-3.5 hr	+ 14.2	- 6.3
1.0 mg/L-6.0 hr	+ 10.5	- 6.0

[a] All animals were exposed for the indicated number of hours per day, 4 days/wk, for 4 wk.

postexposure weight minus its first week pre-exposure weight. The weight gain over the last 3 wk was also compared among modular-exposed and whole-body exposed animals in a separate analysis. An analysis of the whole-body exposures (Figure 6) indicated no significant difference between the Air-NC, Air-WB, and 0.3-WB groups. Each of these groups, however, registered significantly greater weight gains than the 2.0-WB group regardless of sex. This verified our previous observation that whole-body exposure to 2.0 mg/L, 3.5 hr/day, 4 days/wk, for 4 wk resulted in significantly lower body weight than in comparable controls, whether or not the controls are actually put in chamber. Weekly weight gains for these same animals were also analyzed and showed that for each week of exposure the weight gain in the 2.0-WB group was significantly less than all the other groups (Figure 7).

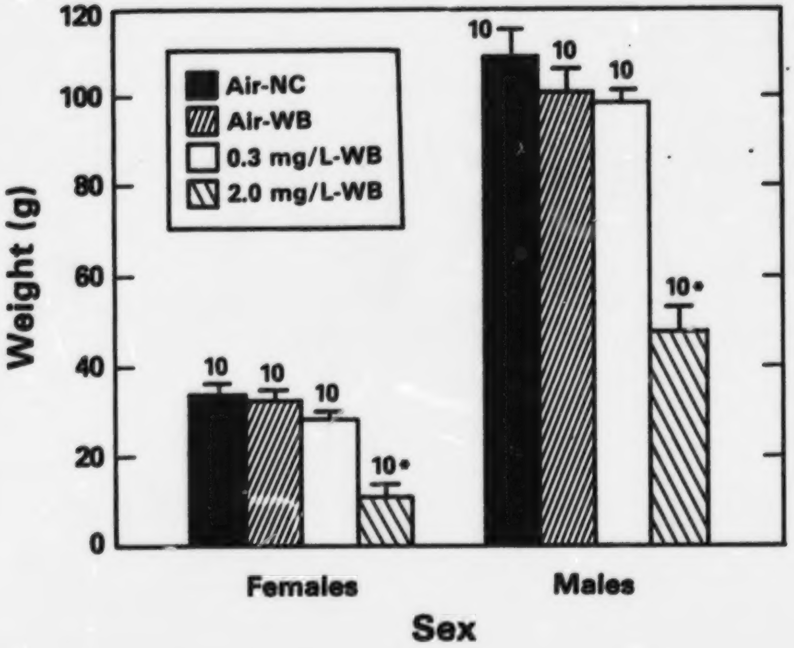

Figure 6. Total weight gain in males and females over 4-wk exposure. NC = no chamber, WB = whole body. Error bars represent the standard error of the mean. *Significantly different from control ($p < 0.05$).

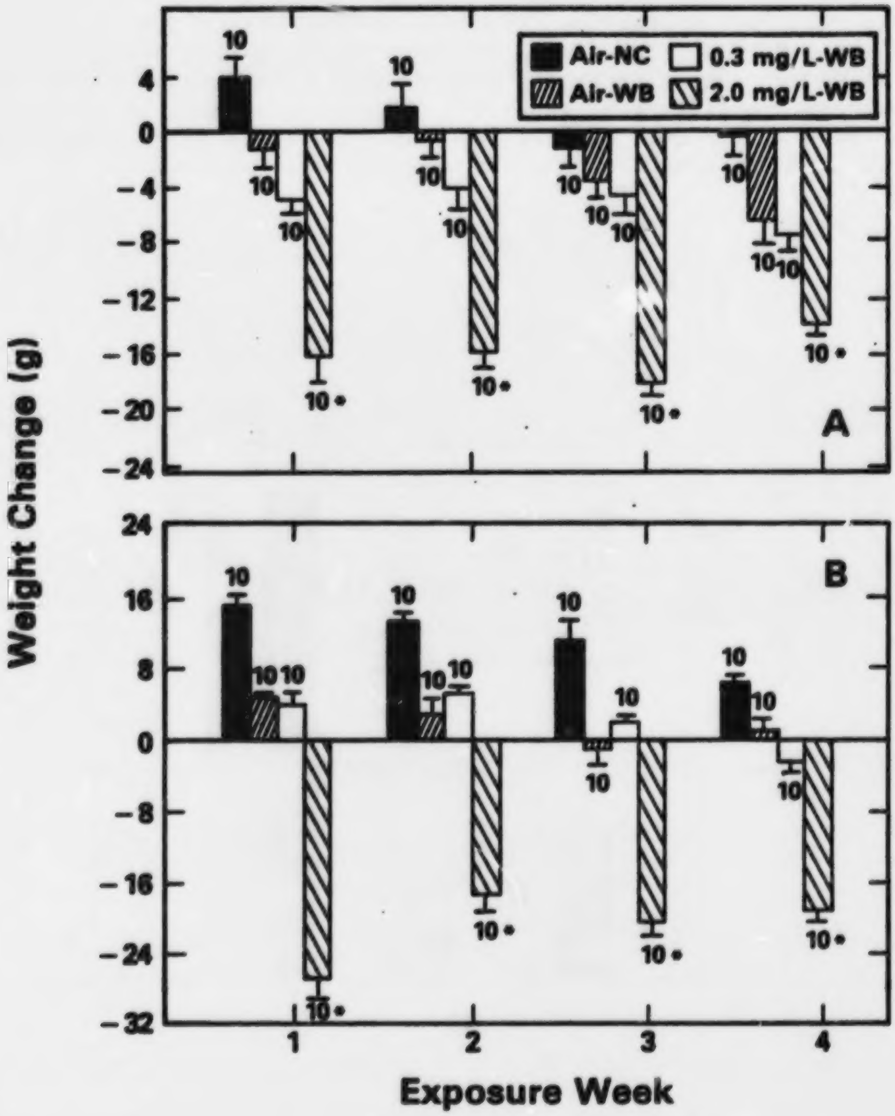

Figure 7. Comparison of weight change over 4 wk by sex. A. Females; B. Males. NC = no chamber, WB = whole body. Error bars represent the standard error of the mean. *Significantly different from control ($p < 0.05$).

Figure 8 shows the comparison of type of exposure (modular vs. whole body) in controls and animals exposed to 2.0 mg/L for the last 3 wk of the experiment. For females, the interaction between type of exposure and concentration was marginally significant (p = 0.08). This appears to be due to the larger difference in weight gain between control and exposed animals in the whole-body groups versus the modular groups. This suggests that in the females some of the weight difference in the whole-body exposure might have been due to ingestion. In males, however, although the effects on weight gain from both types of exposure and from concentration are highly significant (p = 0.0001), there was no interaction between concentration and exposure type. As the figure indicates, the difference between exposed and control animal weights was about the same whether or not the modules were used. This indicated that in the males the difference in weight gain was due to inhalation. It should be noted that the modules used in these experiments were not ideal because the module itself caused a weight loss.

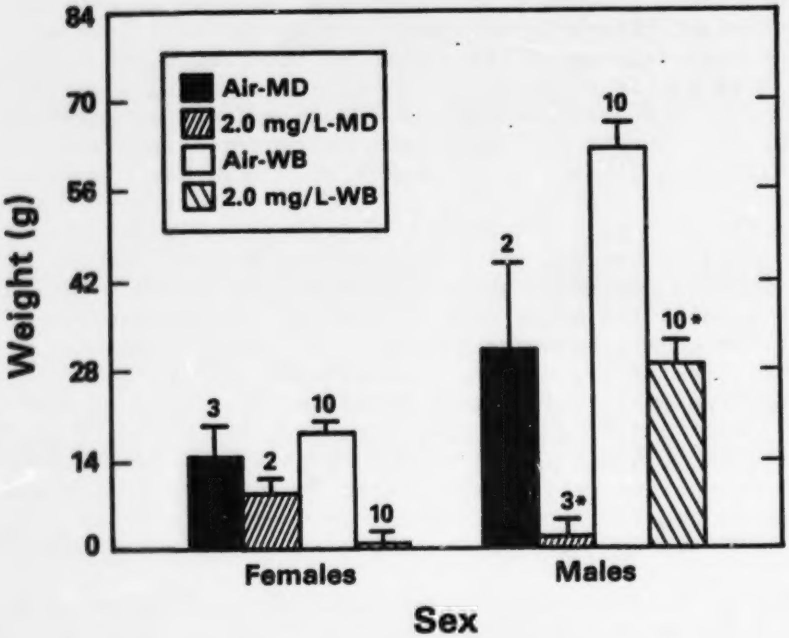

Figure 8. Change in weight over the last 3 wk of exposure comparing modular (MD) and whole-body (WB) groups. Error bars represent the standard error of the mean. *Significantly different from control ($p < 0.05$).

Phase II - Subacute Range-Finding Studies

Male and female rats were exposed for 4 wk in a 3 x 2 x 2 experimental design. The three variables included filtered air, fog oil smoke concentration (0.5 or 1.5 mg/L), frequency of exposures per week (2 or 4 days/wk), and duration of daily exposures (70 min or 3.5 hr). Each biological parameter was tested on these 12 groups except for cardiopulmonary parameters that, for logistical reasons, were tested only at the highest concentration, the longest time and the 4-day/wk frequency. Also, zoxazolamine-induced paralysis time was completed only for the 4-day/wk frequency because of technical difficulties. All measurements were made on the day after the last exposure.

Body Weight

Two sets of data were collected. The weights of one subset of six animals per group were measured immediately before the first and after the last exposure for each of the 4 wk. Secondly, weights of all the animals were routinely measured before the first and after the final exposure in the study. Weight change during each of the 4 wk was examined in both a multivariate and univariate three-way ANOVA. The multivariate ANOVA indicated that the mean vector of weight changes was significantly ($p = 0.0001$) affected by exposure concentration, frequency, and duration. The univariate analyses of each weekly weight change showed that, during the last 3 wk of exposure, the same effects were significant as in the multivariate case. The analysis of the first week's weight change indicated a marginally significant exposure time effect ($p = 0.06$) and a significant concentration by frequency interaction ($p = 0.03$). During the first week, although weight gain decreased with increasing fog oil smoke concentration, there was a much greater difference between the air control group and the 1.5 mg/L group, for animals exposed 4 days/wk (22.3 g), than for those exposed 2 days/wk (4.8 g). For the remaining 3 wk, weight gains even in the 0.5-mg/L group were significantly lower than in controls, pooling over frequency and duration of exposure. Figure 9 shows the effects of the different exposure regimens on weight gain during the weekly exposure periods. In every case, weight gain decreased as concentration of fog oil smoke increased. By examining Figure 9 (A-D), it is also apparent that weight gain decreased when time of exposure was increased from 70 min to 3.5 hr. By comparing parts A through D of Figure 9 one can see that weight gain was less in the 2-day/wk frequency compared to the 4-day/wk frequency. Since weights were taken immediately before and after exposure, Figures 9A and 9B represent weight gain across a 2-day period, whereas Figures 9C and 9D represent weight gain across a 4-day period.

The total weight change from just prior to the first exposure until immediately after the last exposure was examined in all animals. This analysis indicated a significant three-way interaction (Figure 10). Analyzing each frequency group separately, animals exposed 2 days/wk experienced significant weight changes due to the effects of both fog oil smoke concentration and exposure time. These effects appeared to be additive for this frequency group. For animals exposed 4 days/wk, weight change was significantly affected by the interaction of fog oil smoke concentration and

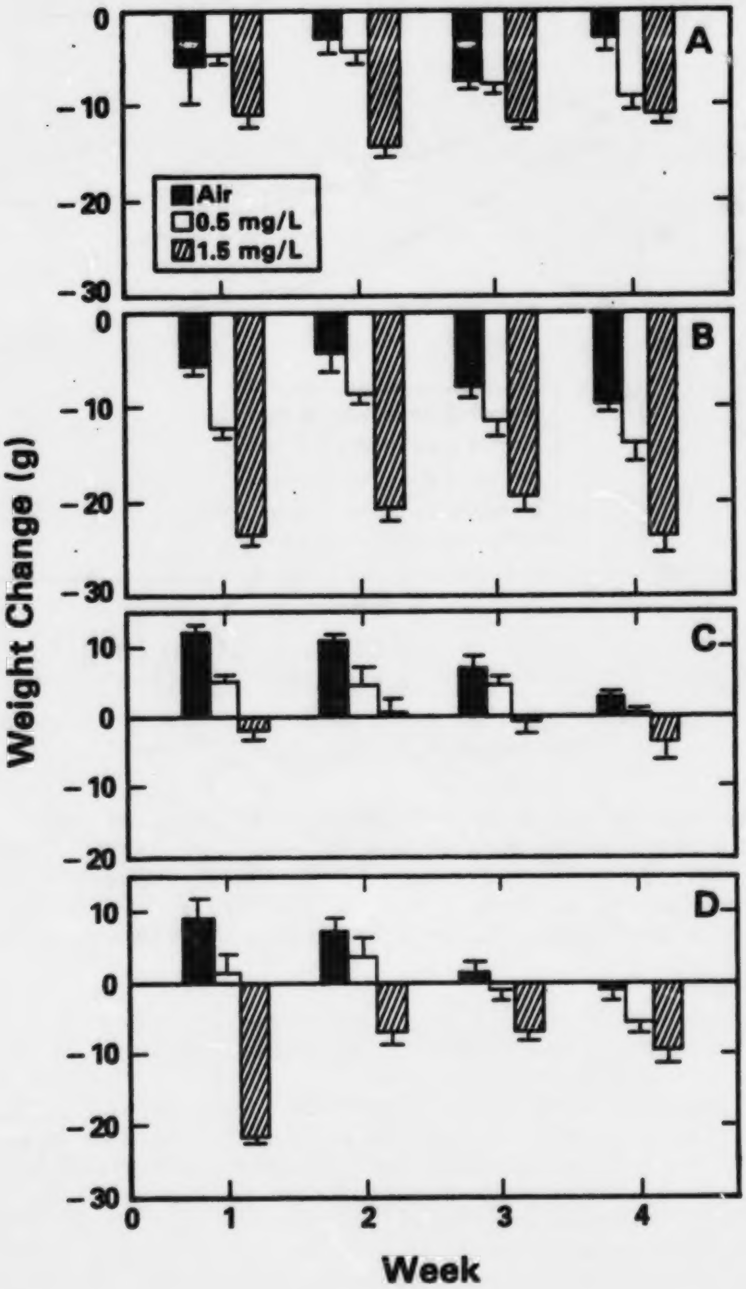

Figure 9. Body weight changes over 4 wk by exposure frequency and time. A. 2 days/wk-70 min; B. 2 days/wk-3.5 hr; C. 4 days/wk-70 min; D. 4 days/wk-3.5 hr. Error bars represent the standard error of the mean.

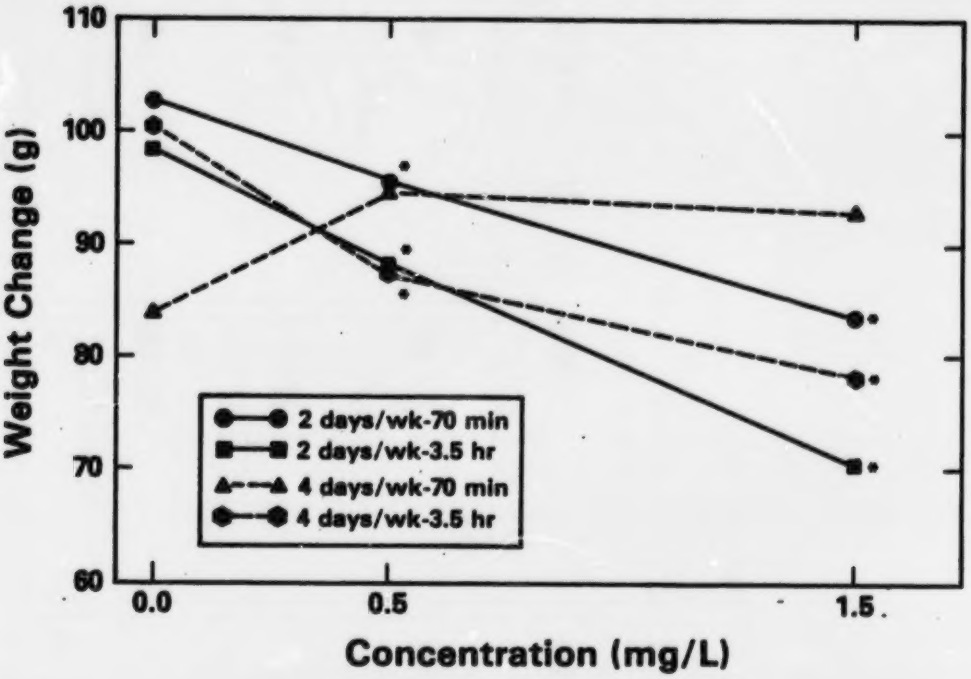

Figure 10. Weight change over the entire 4-wk study. *Significantly different from control ($p < 0.05$).

exposure time ($p = 0.0002$). Least squares means of weight gain for animals exposed 4 days/wk for 70 min were 84, 94, and 92 g by increasing concentrations of fog oil smoke. For animals exposed 4 days/wk for 3.5 hr, the corresponding means were 100, 87, and 78 g. Weight gain was much more variable in the control group than in other treatment groups.

Histopathology

Histopathology was conducted as summarized in Table 10.

For Exposure Group A, no significant microscopic findings were observed in the nasal cavity, eyes, heart, skin, larynx, trachea, kidney, liver, stomach, duodenum, testes, or epididymides. However, a few incidental findings such as focal chronic myocarditis, focal tubular regeneration in the kidney, unilateral seminiferous tubular atrophy and a corresponding lack of sperm in the epididymides were observed occasionally. Focal hemorrhage, histiocytosis, and focal hemosiderosis were observed occasionally in peribronchial lymph nodes from all concentration groups.

Histopathologic findings were similar in the right diaphragmatic and left lobes of the lung. A minimal amount of peribronchial lymphocytic infiltrate

TABLE 10. HISTOPATHOLOGY OF PHASE II - SUBACUTE RANGE-FINDING STUDIES

Exposure Group A	Time Exposed	No. of Male Rats
Air (Control)	2 days/wk-70 min	4
0.5 mg/L	2 days/wk-70 min	4
1.5 mg/L	2 days/wk-70 min	4
Air (Control)	4 days/wk-70 min	4
0.5 mg/L	4 days/wk-70 min	4
1.5 mg/L	4 days/wk-70 min	4
Air (Control)	2 days/wk-3.5 hr	4
0.5 mg/L	2 days/wk-3.5 hr	4
1.5 mg/L	2 days/wk-3.5 hr	4
Air (Control)	4 days/wk-3.5 hr	4
0.5 mg/L	4 days/wk-3.5 hr	4
1.5 mg/L	4 days/wk-3.5 hr	4

		No. of Rats	
Exposure Group B	Time Exposed	Male	Female
Air (Control)	4 days/wk-3.5 hr	6	6
0.5 mg/L	4 days/wk-3.5 hr	6	6
1.5 mg/L	4 days/wk-3.5 hr	7[a]	8[a]

[a] In the 1.5-mg/L exposure group, male number 420 and female number 464 died after two exposure periods, and female number 466 died after one exposure period.

was present in most lungs and was considered to be normal. A minimal number of macrophages were scattered throughout the pulmonary alveolar lumens of most animals exposed to fog oil smoke for 70 min. Usually only one macrophage was in an alveolus and these alveolar macrophages contained eosinophilic (protein-like) material in their cytoplasm. One rat exposed for 70 min/day for 4 days/wk exhibited a slight increase in the number of macrophages in the alveolar lumens. The incidence of lungs with lesions was lowest (1 of 4 lungs) for rats exposed 70 min/day for 2 days/wk at 0.5 mg/L. Pulmonary lesions in most rats exposed for 3.5 hr at 0.5 or 1.5 mg/L were similar to those noted in rats exposed for 70 min and consisted of macrophages in the alveolar lumens. However, the number of macrophages in the alveoli was greater in the group exposed for 3.5 hr/day for 4 days/wk at 1.5 mg/L.

For Exposure Group B, treatment-related microscopic changes were observed in the lungs of male and female rats of both concentration groups. These changes included a diffuse accumulation of macrophages within the alveoli of exposed male and female rats. The degree of severity was concentration related with slight to moderate involvement in the 1.5-mg/L exposure group and minimal to slight involvement in the 0.5-mg/L exposure groups. In affected rats, scattered alveolar macrophages were present throughout the lung. In addition, four of six male rats at the 1.5-mg/L exposure level also had slight to moderate multi-focal pneumonitis. In these rats, multifocal hypercellularity of the alveolar wall, associated with an interstitial infiltration of subacute inflammatory cells, was observed. In addition to the accumulation of macrophages, polymorphonuclear inflammatory cells were also present within the alveoli of male rats with pneumonitis. Other changes that were observed within the lung were considered to be incidental and most frequently included peribronchial and perivascular lymphoid infiltrates.

No treatment-related changes were present in other tissues examined in this study. A few incidental lesions such as chronic myocarditis, tubular regeneration in the kidney, and seminiferous tubular atrophy in the testes were occasionally observed. These changes were considered to be within normal limits for rats of this age and strain.

Pulmonary Physiology

The following parameters were analyzed: diffusing capacity of carbon monoxide (DL_{co}), total lung capacity (TLC), vital capacity, end expiratory volume (EEV), nitrogen (N_2) washout slope, N_2 washout corrected for changes in EEV (CEVSLP), lung wet weight, lung dry weight, and body weight. Multivariate analysis showed effects due to time and concentration but not due to frequency. There was also a CONC by TIME interaction. Univariate analysis of the individual parameters showed significant effects in lung wet weight, lung dry weight, and EEV (Figures 11-13). For both lung weights there was a significant concentration effect and CONC by TIME interaction. Lung weight in the 1.5-mg/L exposure group increased significantly compared to that of the air control group at the 3.5-hr duration time regardless of frequency. At the 3.5-hr/day and 4-day/wk exposure, EEV showed a concentration response. The 1.5-mg/L treatment for this regimen was significantly different than all other treatment groups. EEV of the 0.5-mg/L group was significantly elevated compared to that of the air control group for that particular regimen. In groups exposed for 3.5 hr/day, 2 days/wk, there was a trend toward a concentration-related increase in EEV, but no statistically significant changes were observed. No significant effects were found in the other pulmonary physiology parameters (Figures 14-19).

Results for the biological parameters from the second replicate data set were compared to those from the first experiment to examine the reproducibility of the test results. A variable, REP, was assigned a value of one if the data arose from the first study or a value of two if it was produced in the second study. A two-way ANOVA model was then fit to the combined data set containing the response variables of primary interest. A significant CONC by REP interaction indicated nonreproducibility among replicates.

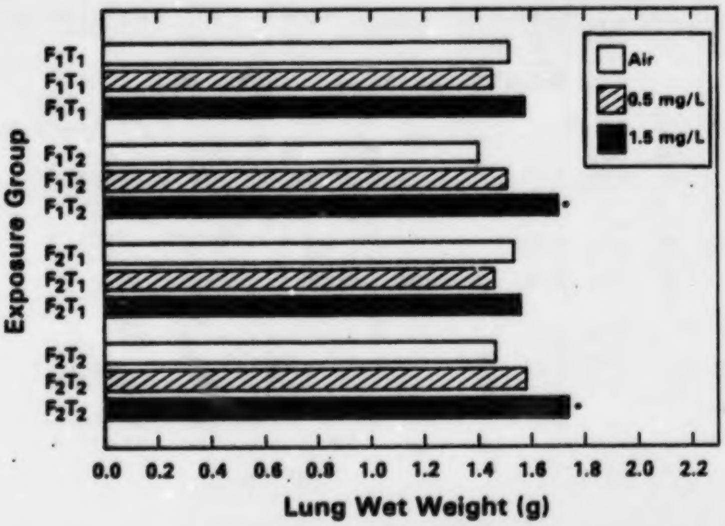

Figure 11. Effect of 4-wk exposure to fog oil smoke on lung wet weight. $F_1 = 2$ days/wk; $F_2 = 4$ days/wk; $T_1 = 70$ min/day; $T_2 = 3.5$ hr/day. *Significantly different from control ($p < 0.05$).

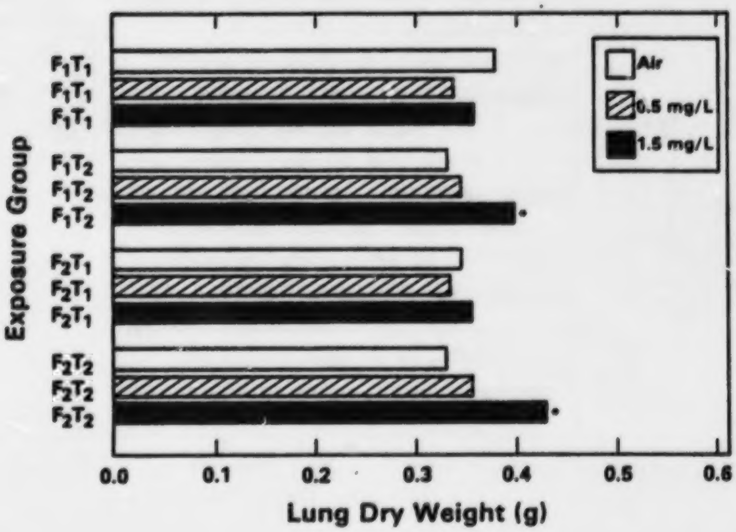

Figure 12. Effect of 4-wk exposure to fog oil smoke on lung dry weight. $F_1 = 2$ days/wk; $F_2 = 4$ days/wk; $T_1 = 70$ min/day; $T_2 = 3.5$ hr/day. *Significantly different from control ($p < 0.05$).

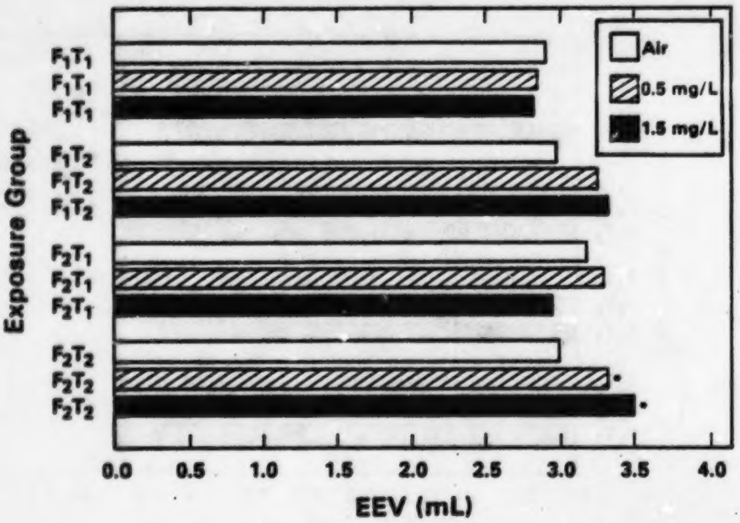

Figure 13. Effect of 4-wk exposure to fog oil smoke on end expiratory volume (EEV). $F_1 = 2$ days/wk; $F_2 = 4$ days/wk; $T_1 = 70$ min/day; $T_2 = 3.5$ hr/day. *Significantly different from control ($p < 0.05$).

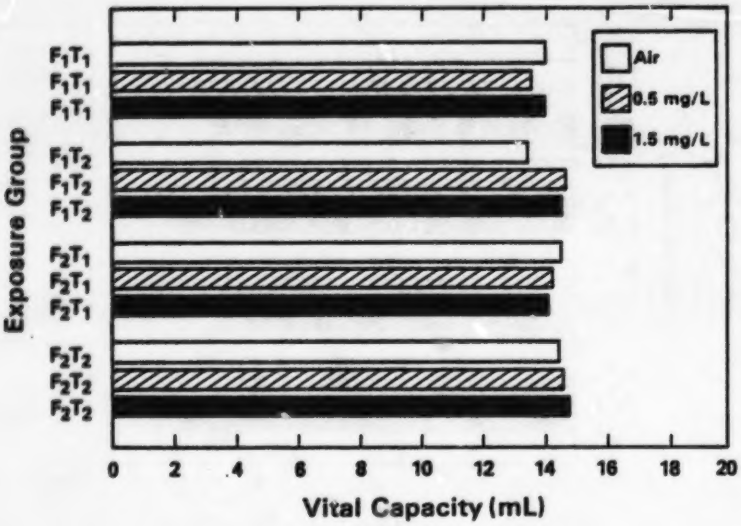

Figure 14. Effect of 4-wk exposure to fog oil smoke on vital capacity. $F_1 = 2$ days/wk; $F_2 = 4$ days/wk; $T_1 = 70$ min/day; $T_2 = 3.5$ hr/day. *Significantly different from control ($p < 0.05$).

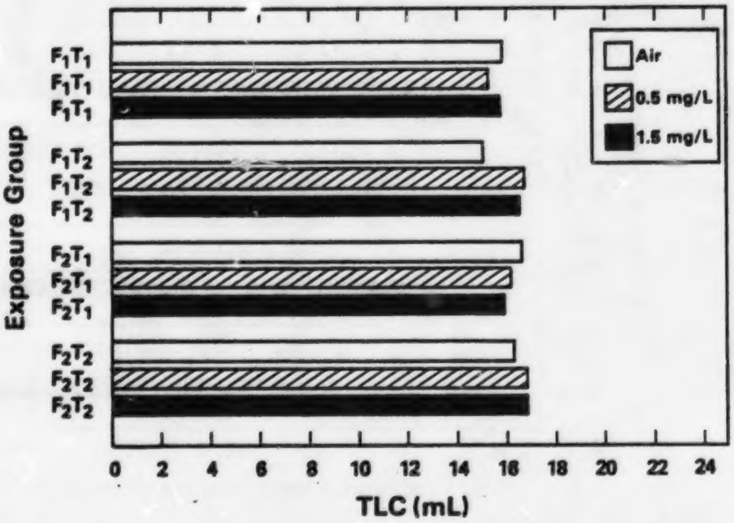

Figure 15. Effect of 4-wk exposure to fog oil smoke on total lung capacity (TLC).
$F_1 = 2$ days/wk; $F_2 = 4$ days/wk; $T_1 = 70$ min/day; $T_2 = 3.5$ hr/day.

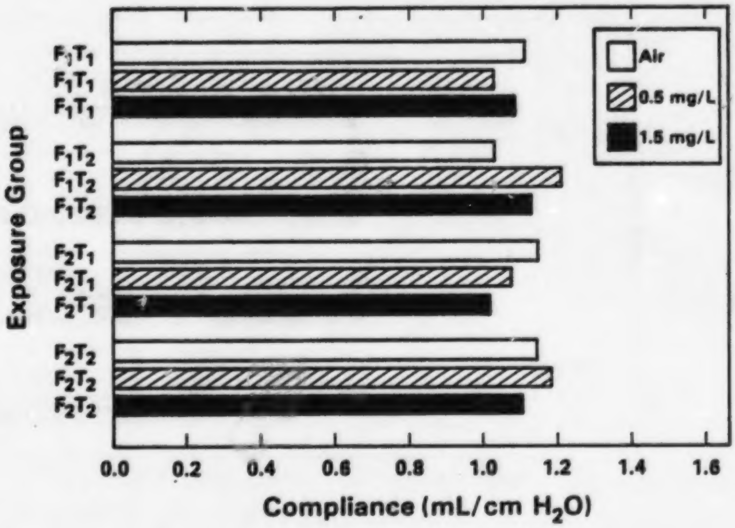

Figure 16. Effect of 4-wk exposure to fog oil smoke on compliance.
$F_1 = 2$ days/wk; $F_2 = 4$ days/wk; $T_1 = 70$ min/day; $T_2 = 3.5$ hr/day.

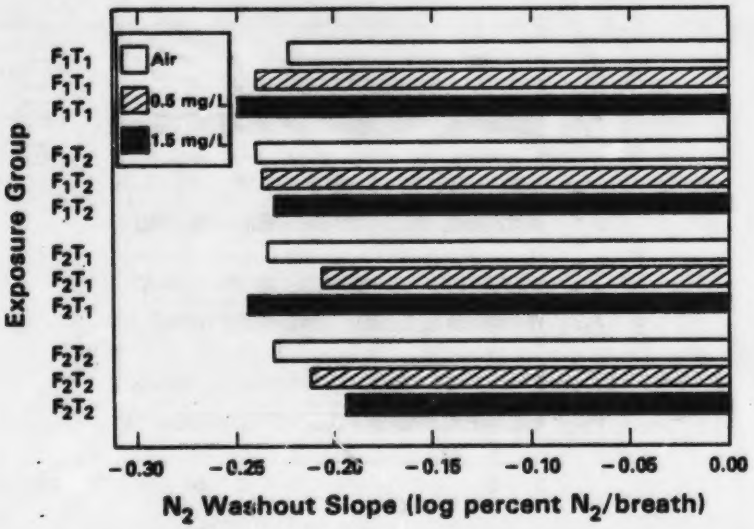

Figure 17. Effect of 4-wk exposure to fog oil smoke on nitrogen (N_2) washout slope. F_1 = 2 days/wk; F_2 = 4 days/wk; T_1 = 70 min/day; T_2 = 3.5 hr/day.

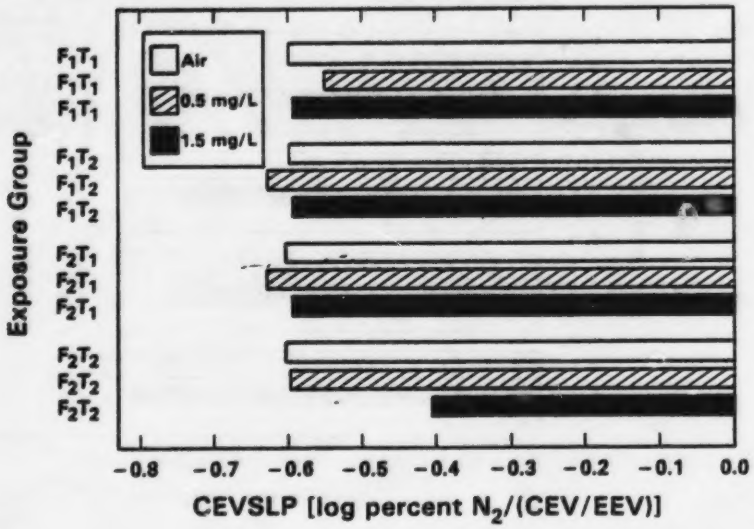

Figure 18. Effect of 4-wk exposure to fog oil smoke on nitrogen (N_2) washout slope corrected for EEV (CEVSLP). CEV = Cumulative expiratory volume. F_1 = 2 days/wk; F_2 = 4 days/wk; T_1 = 70 min/day; T_2 = 3.5 hr/day.

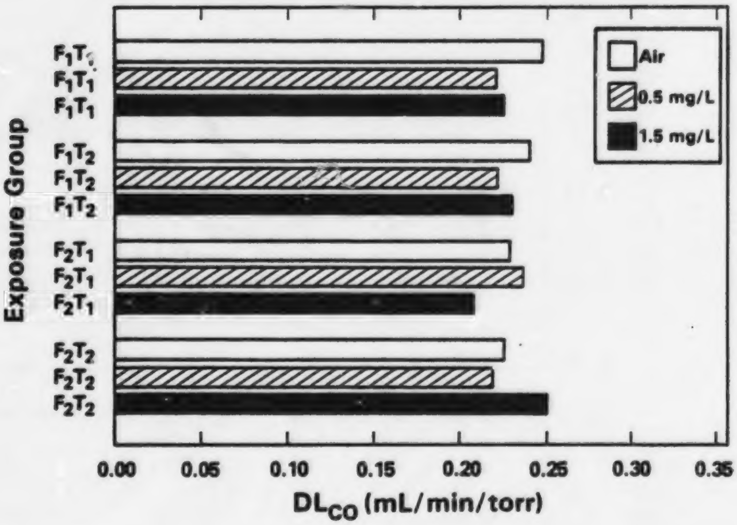

Figure 19. Effect of 4-wk exposure to fog oil smoke on diffusing capacity (DL_{CO}). $F_1 = 2$ days/wk; $F_2 = 4$ days/wk; $T_1 = 70$ min/day; $T_2 = 3.5$ hr/day.

When the responses were examined in a two-way multivariate ANOVA, there was no indication of a significant REP by CONC interaction with respect to the aggregate response. Significant probabilities ranged from 0.23 to 0.24, depending on the particular test statistic employed (Williams' or t tests). The multivariate tests of REP and CONC were both statistically significant regardless of the particular test statistic. Univariate ANOVA models were fit to each of the dependent variables to see which variable responses were most and least affected in terms of reproducibility and overall concentration response. In no case was a significant REP by CONC interaction detectable. For the DL_{CO}, TLC, N_2 slope (log of the percentage of N_2/breath), vital capacity, EEV, lung dry weight, lung wet weight, and body weight, there was a significant REP effect ($p < 0.01$). In the absence of a REP by CONC interaction, however, this does not mean that these results were not reproducible from replicate to replicate, but only that the concentration-response pattern shifted noticeably up or down.

Lung dry weight ($p = 0.0001$), lung wet weight ($p = 0.0002$), and EEV ($p = 0.006$) were the only responses that showed significant concentration-related effects pooled over both replicates (Figures 20 and 21). Individual contrasts of mean control versus mean response at each of the two fog oil smoke concentrations indicated that for EEV, the only significant difference occurred between controls and the high concentration ($p = 0.002$). The same situation occurred for lung dry ($p = 0.0001$) and wet ($p = 0.0001$) weights. None of the parameters tested showed significant effects at the 0.5-mg/L concentration.

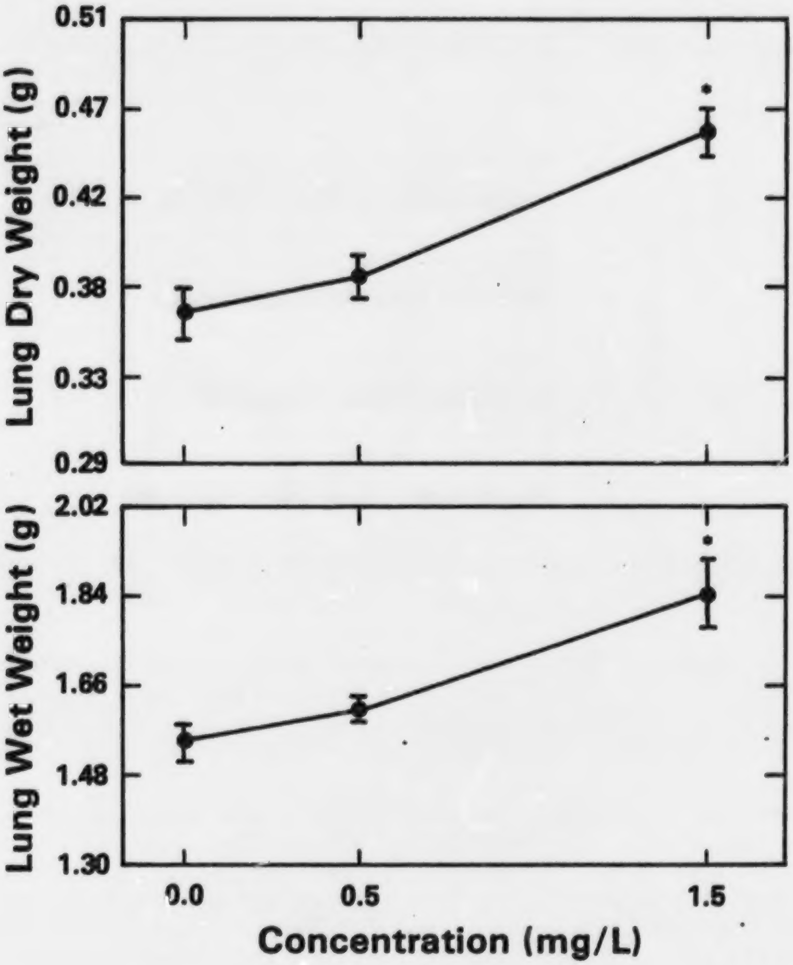

Figure 20. Pooled replicate effect of 4-wk exposure to fog oil smoke on lung wet and dry weights. Error bars represent the standard error of the mean. *Significantly different from control ($p < 0.05$).

Cardiopulmonary Physiology

Two groups of 12 rats were obtained on different days. Of those 12 animals, half were air controls, and half were exposed to 1.5 mg/L fog oil smoke, 3.5 hr/day, 4 days/wk for 4 wk. Of the 24 animals, 16 were used to study pulmonary function, and the remaining 8 were used for cardiovascular evaluation. Six animals in the pulmonary function group and two of the eight cardiovascular group rats could not be used because of surgical failures. The data from five controls and five treated animals revealed no significant differences in pulmonary function after statistical analysis using t tests. Although not statistically significant, the data suggested increased expiratory resistance in treated animals that may have caused a slight elevation in breathing rate and intrapleural pressure.

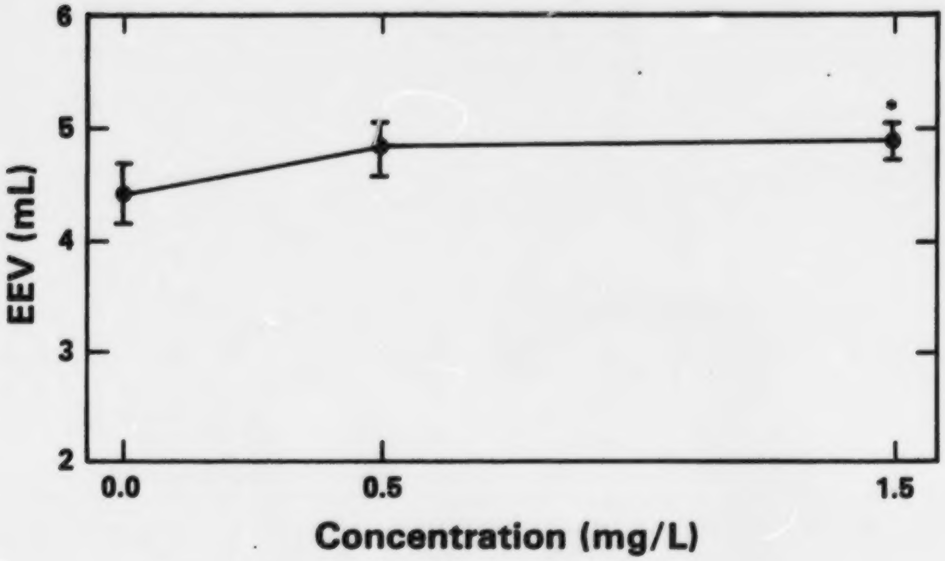

Figure 21. **Pooled replicate effect of 4-wk exposure to fog oil smoke on EEV. Error bars represent the standard error of the mean. *Significantly different from control ($p < 0.05$).**

Because only three treated animals and three controls were examined for cardiovascular effects, results were inconclusive. Originally, an additional group of 12 animals (6 exposed and 6 unexposed) were to be provided for these parameters. However, this would have involved running two chambers for 4 wk with only six animals each. We decided not to do this and the project officer concurred.

Pulmonary Edema

The main response of interest in this end point is protein concentration in the cell-free, lung lavage fluid. The logs of protein level were fit to a saturated three-way ANOVA model. The analysis detected no significant interactions involving main effect due to exposure duration but did show a significant ($p = 0.039$) exposure frequency by exposure concentration interaction (Figure 22). Post hoc t tests of the differences in least square means indicated that protein levels in rats exposed to 1.5 mg/L of fog oil smoke, 4 days/wk, were significantly elevated over control response ($p = 0.0001$) and also significantly elevated ($p = 0.007$) in comparison to the mean level in animals exposed to the same fog oil smoke concentration, but for only 2 days/wk.

In regard to the reproducibility of replicates 1 and 2, the first replicate showed mean lavage fluid protein concentration to be elevated approximately 8% by the 0.5-mg/L exposure and approximately 72% by the 1.5-mg/L level (Figure 22). The second experiment had a higher basal lavage fluid protein concentration but showed somewhat the same pattern: little or

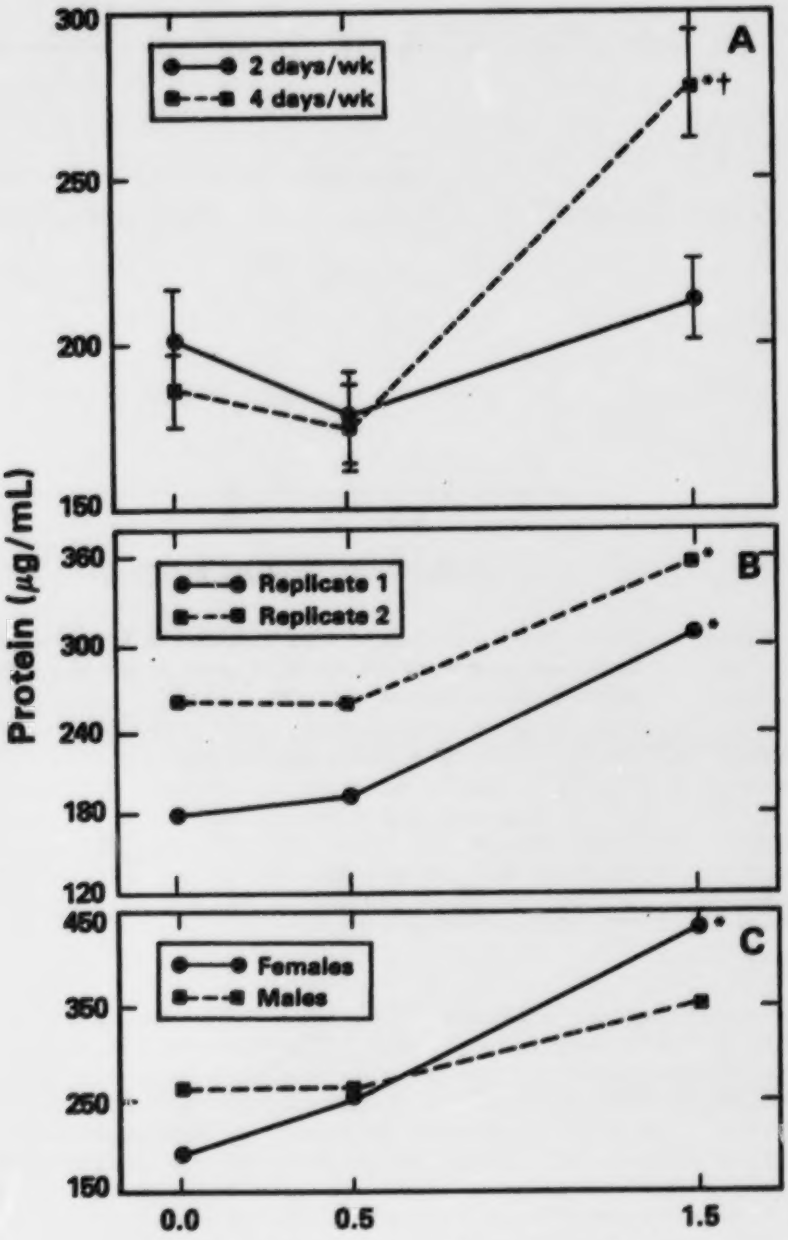

Figure 22. Effects of 4-wk exposure to fog oil smoke on lavage fluid protein. A. Combined analysis by frequency of exposure; B. Replicate analysis; C. Female/male comparison. Error bars represent the standard error of the mean. *Significantly different from control ($p < 0.05$). †Significantly different from 2 days/wk at 1.5 mg/L.

no elevation at the 0.5-mg/L exposure level and a 36% increase at the 1.5-mg/L exposure level. Univariate ANOVA of the data from both replicates indicated no interactions that would prohibit pooling of data from both replicates. When this was done, appropriate contrasts indicated a significant elevation of lavage fluid protein concentration at the 1.5-mg/L exposure level (p = 0.0023) but not at the 0.5-mg/L level of exposure.

Because both sexes were exposed in the second replicate, a possible difference between the response of male and female rats was investigated using two-way ANOVA on the data of this replicate only. No effects involving gender were detected, indicating that the response of males and females was statistically indistinguishable (Figure 22). When data from males and females were pooled, the increase over control at 1.5 mg/L in lavage fluid protein concentration was significant, as was the increase seen when pooled data from the two replicates were analyzed.

Pulmonary Cells

Total cell counts, cell viability determinations, and differential cell counts were performed on cells from pulmonary lavage fluid. The parameters of interest (total cells, percentage of viability of total cells, and percentages of macrophages, lymphocytes, polymorphonuclear leukocytes [PMNs] and eosinophils) were examined with univariate saturated three-way ANOVA models. Total cells were log-transformed, and percentages of PMNs and eosinophils were arc sine square root transformed to satisfy better the usual ANOVA assumptions. There was still evidence of considerable non-normality of the latter two percentages, however, even after such transformation. Post hoc subtests of standardized differences in least squares means comparing control group mean versus each fog oil smoke concentration group mean were performed for each combination of exposure frequency and exposure duration. Thus, eight such tests were performed on each of the above parameters. To hold the overall Type I error to 5%, only the results of such tests that were significant at the 0.05/8 = 0.006 probability level are cited here as significant. The ANOVA of logs of total cells indicated significant main effects of exposure frequency (p = 0.0001), exposure duration (p = 0.04), and exposure concentration (p = 0.04). The only subtest that was significant at the adjusted probability level was the difference between control and the 1.5-mg/L concentration group means for animals exposed 4 days/wk for 3.5 hr (Figure 23). No other such test even approached significance. Thus, it appears that for the worst case exposure there was an influx of cells into the lung. The ANOVA of the percentage of viability indicated a significant three-way interaction (p = 0.01) between frequency, duration, and concentration. The post hoc t test of the control means versus the 1.5-mg/L means for the 2-day/wk-70 min exposure group was significant (p = 0.0001) with means of 66% and 89%, respectively. This result appeared to be due to abnormally low values for the control and 0.5-mg/L groups (Figure 23). The only other test approaching significance (p = 0.02) was the test of the difference in means between controls (85%) and the 1.5-mg/L group (92%) in animals exposed 4 days/wk for 3.5 hr.

ANOVA of the percentage of macrophages also indicated a three-way interaction (p = 0.02) between the three factors considered. Examining the post hoc t test results, only the difference between the control group mean of 51% and the 0.5-mg/L group mean of 75% in the 4-day/wk-3.5-hr group approached the adjusted significance level with a probability of 0.0125 (Figure 23).

The interaction of concentration and time was significant (p = 0.001) for the percentage of PMNs. There was no indication of an interactive or main effect involving exposure frequency. Of the post hoc tests of differences of least square means, two were significant at the p = 0.0001 level (Figure 24):

1. the control mean of 0.6% versus the 1.5-mg/L mean of 16.9% for rats exposed 2 days/wk-3.5 hr/day, and

2. the control mean of 2.4% versus the 1.5-mg/L mean of 15.1% for rats exposed 4 days/wk-3.5 hr/day.

With respect to the percentage of lymphocytes, the three-way interaction was again significant (p = 0.002). Three of the t tests were significant (Figure 24) at the adjusted significance level:

1. the control group mean of 53% versus 13% for the 1.5-mg/L group for animals exposed 2 days/wk-3.5 hr/day

2. the control group mean of 47% versus a 0.5-mg/L group mean of 18% for rats exposed 4 days/wk-3.5 hr/day

3. the control group mean of 47% versus the 1.5-mg/L group mean of 23% for rats exposed 4 days/wk-3.5 hr/day.

The percentage of eosinophils showed a marginally significant three-way interaction (p = 0.07) in the ANOVA. Only one of the post hoc tests was significant: the difference between the control mean of 0.0% and the 1.5-mg/L mean of 0.8% in animals exposed for 3.5 hr, 4 days/wk for 4 wk. (Figure 24). Significance levels for this variable should be viewed with extreme caution, however, due to heteroscedasticity and non-normality problems.

Figure 25 compares the Phase II replicates for pulmonary cell counts. The combined replicate analyses indicated a significant increase in total cells following a 1.5-mg/L exposure. This appears to be due to a significant (p = 0.0001) influx of PMNs (Figure 25), which was significant for each replicate and when the replicates were combined. The combined replicate analysis also indicated a significant (p = 0.030) increase in total cells at 0.5 mg/L, but the percentage of PMNs was not significantly increased at 0.5 mg/L. Viability of the pulmonary cells (Figure 25) appeared to be significantly enhanced by the fog oil smoke treatment, at both the 0.5-mg/L and 1.5-mg/L concentrations; however, there was a significant interaction between replicates. Effects were actually greater at 0.5 mg/L than at 1.5 mg/L.

The percentage of alveolar macrophages recovered decreased with increasing concentration when replicates 1, 2, and 3 were combined (Figure 25). There was a significant interaction between REP and CONC but this was probably due to the unusually low control value for the first replicate. The decrease in

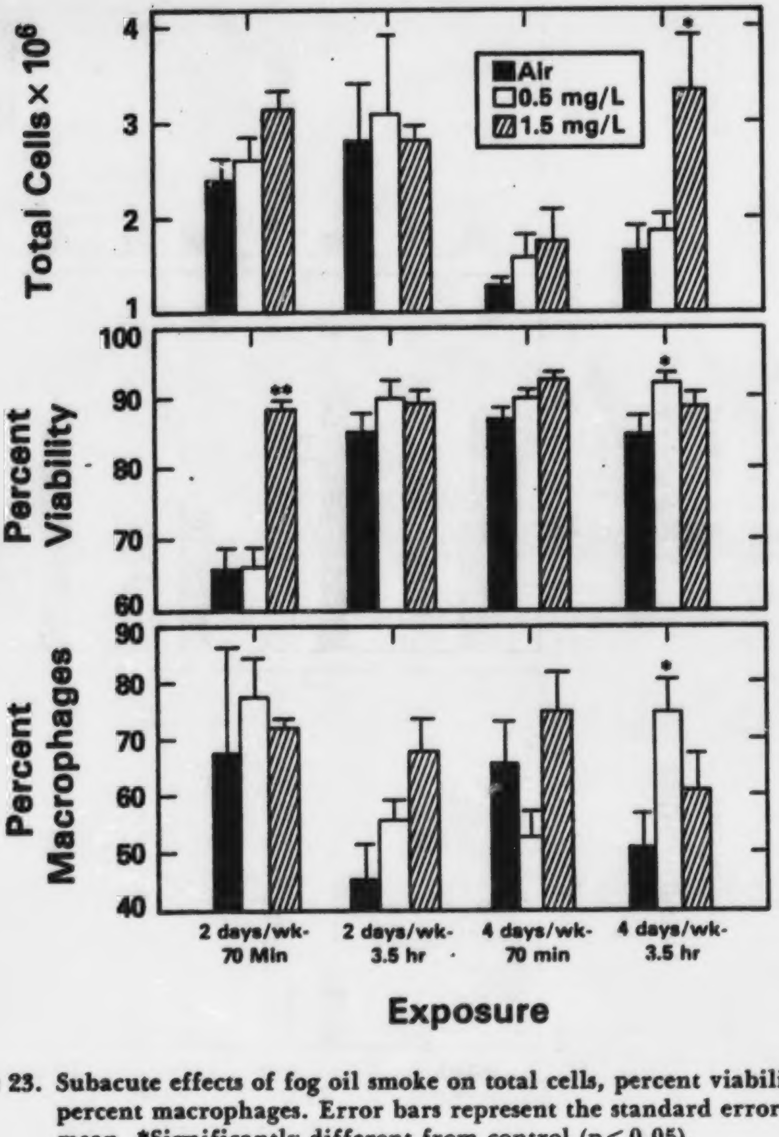

Figure 23. Subacute effects of fog oil smoke on total cells, percent viability, and percent macrophages. Error bars represent the standard error of the mean. *Significantly different from control ($p < 0.05$). **Significantly different from control ($p < 0.01$).

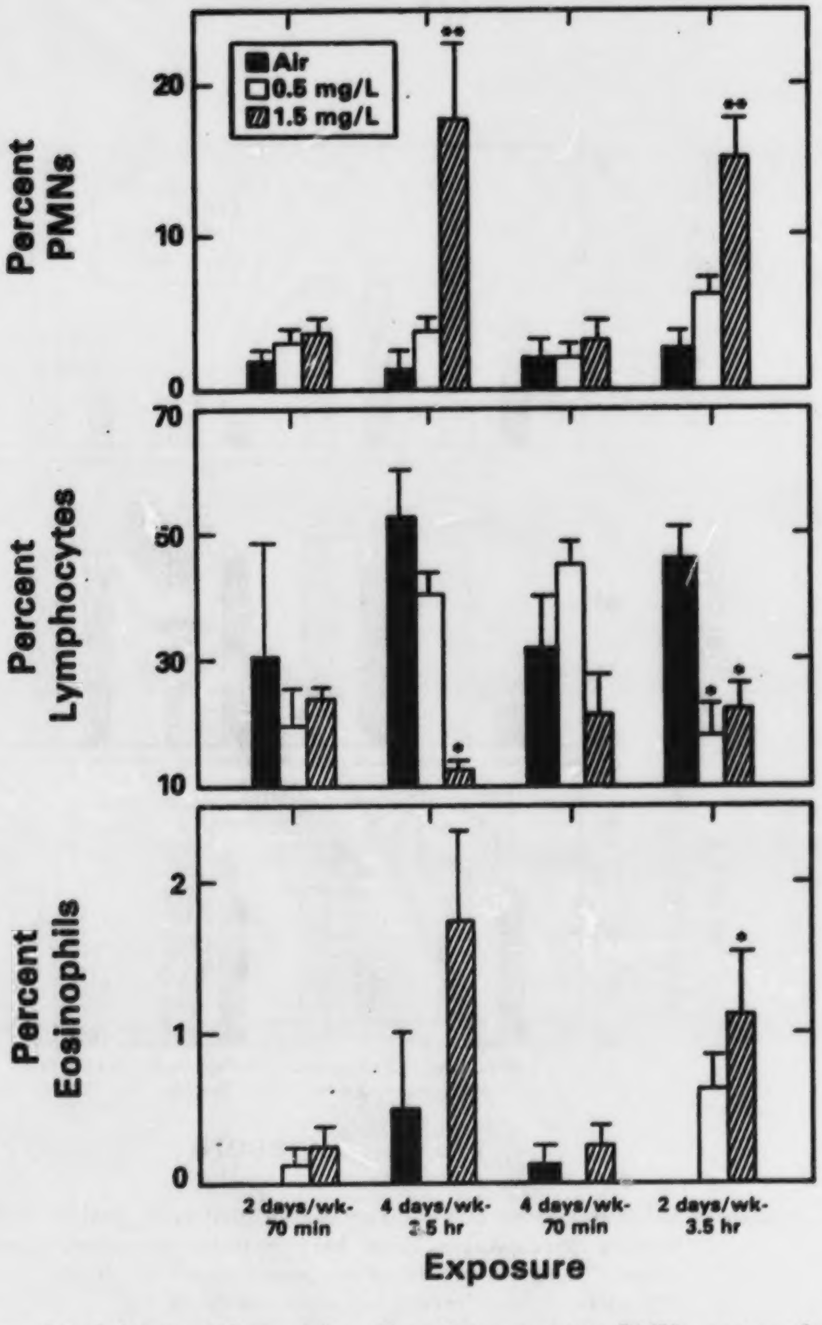

Figure 24. Subacute effects of fog oil smoke on percent PMNs, percent lymphocytes, and percent eosinophils. Error bars represent the standard error of the mean. *Significantly different from control ($p < 0.05$). **Significantly different from control ($p < 0.01$).

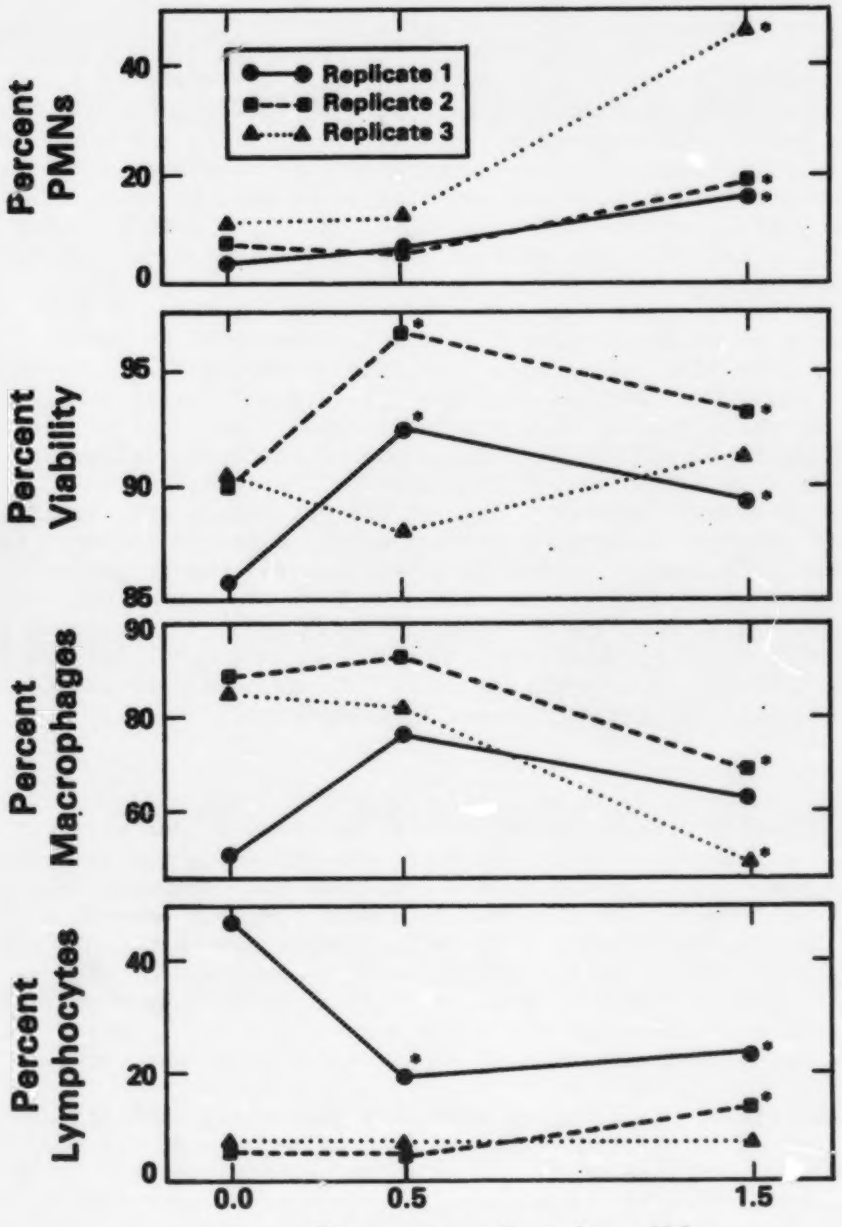

Figure 25. Replicate pulmonary cell response to 4-wk fog oil smoke exposure on percent PMNs, percent viability, percent macrophages, and percent lymphocytes. *Significantly different from control ($p < 0.05$).

percentage of alveolar macrophages corresponded to an increase in percentage of PMNs. The percentage of lymphocyte replicates indicated a variety of responses (Figure 25). Based on the individual and combined analyses, there was no effect due to fog oil smoke on the percentage of lymphocytes.

Behavioral Response

Activity of rats in a figure-eight maze was recorded via eight photodiode pairs mounted in the maze. A saturated three-way ANOVA model was initially fit to these activity counts. However, to satisfy the usual ANOVA assumptions better, logarithms of these counts were also analyzed. The latter analysis indicated that the three-way interaction between exposure frequency, concentration, and duration was significant ($p = 0.0085$), thus precluding our ability to look at tests of main effects or lower-order interactions within the framework of this model. To explore the nature of this interaction, separate two-way ANOVAs were run on the logs of the activity counts for each frequency of exposure (2 or 4 days/wk). The ANOVA of counts for those animals exposed 2 days/wk indicated a significant CONC by TIME interaction ($p = 0.02$, Figure 26). Post hoc tests of the standardized difference in least square means for rats exposed 70 min and for rats exposed 3.5 hr, at each fog oil smoke concentration, indicated the most significant difference occurred in the controls ($p = 0.03$) exposed to air rather than fog oil smoke. The two-way ANOVA for animals exposed 4 days/wk did not detect any significant interaction or main effect. In fact, a pattern nearly opposite to those exposed 2 days/wk occurred. This was undoubtedly the reason for the three-way interaction. Comparison of replicate 1 to replicate 2 of the 3.5-hr/day, 4-day/wk, 4-wk experiment showed a marked variability making it difficult to interpret, especially with regard to the 0.5-mg/L exposure level (Figure 26). At the 1.5-mg/L level, there appeared to be a trend toward increased activity; however, this was not statistically significant.

Clinical Chemistry

Both multivariate and univariate three-way ANOVAs were performed. A significant amount of variance heterogeneity among the different treatment groups was evident. No standard transformation helped. Data were therefore rank transformed. The same analyses were then run on both the untransformed and rank-transformed data. The multivariate analyses could not detect any significant effect due to fog oil smoke concentration. The results shown in Table 11 were at least marginally significant for the univariate analyses upon examination of the eight contrasts comparing controls versus fog oil smoke groups for each combination of exposure frequency and exposure duration.

Analysis of the clinical chemistry parameters combined from replicates 1 and 2 showed no statistically significant changes due to treatment (Figures 27 and 28). From a qualitative standpoint, examination of the means, pooled over replicates, revealed a monotonic increase or decrease with respect to fog oil smoke concentration for albumin, cholesterol, bilirubin, cholinesterase, leucine aminopeptidase, inorganic phosphorous, and protein. Because there is a 33% chance of seeing a monotonic increase or decrease in the examination of mean responses at three concentrations, and because we were examining 20 variables, we would expect 6 or 7 variables to show such a trend by chance.

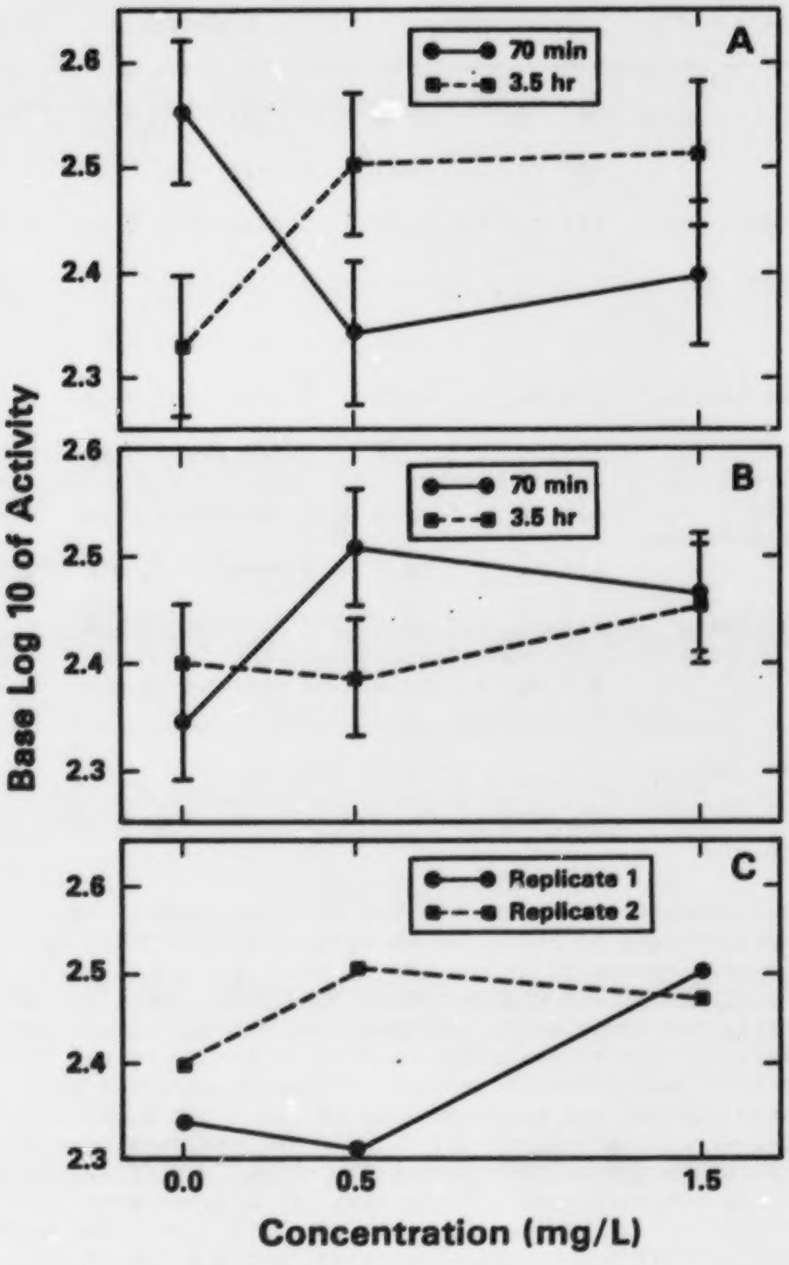

Figure 26. Effects of 4-wk fog oil smoke exposure on rat behavior as measured with a figure-eight maze. A. Combined 2 days/wk analysis by time; B. Combined 4 days/wk analysis by time; C. Replicate analysis. Error bars represent the standard error of the mean.

TABLE 11. EFFECT OF FREQUENCY OF FOG OIL SMOKE EXPOSURE ON CLINICAL CHEMISTRY PARAMETERS

Variable	Contrast	Exposure	p	Critical p[a]
Albumin	Air vs. 0.5 mg/L[a]	2 days/wk-3.5 hr	0.018	0.006
	Air vs. 1.5 mg/L[a]	2 days/wk-3.5 hr	0.018	0.006
Aldolase	Air vs. 0.5 mg/L	2 days/wk-3.5 hr	0.016	0.006
Blood urea N_2	Air vs. 1.5 mg/L[a]	2 days/wk-70 min	0.010	0.006
	Air vs. 0.5 mg/L	2 days/wk-3.5 hr	0.003	0.006
Creatine kinase	Air vs. 0.5 mg/L	2 days/wk-3.5 hr	0.003	0.006
Protein	Air vs. 0.5 mg/L	2 days/wk-3.5 hr	0.033	0.006
Aspartate aminotransferase	Air vs. 0.5 mg/L	2 days/wk-3.5 hr	0.004	0.006
	Air vs. 0.5 mg/L	4 days/wk-70 min	0.0014	0.006
Cholinesterase	Air vs. 0.5 mg/L[a]	2 days/wk-70 min	0.0014	0.006
	Air vs. 1.5 mg/L[a]	2 days/wk-70 min	0.0001	0.006

a. $p = 0.05 \div 8$.

Hematology

Nine variables were examined for this end point: white blood cell count (WBC), percentages of PMNs, percentages of lymphocytes, red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) in erythrocytes, percentage of hematocrit, and hemoglobin. Multivariate and univariate ANOVAs were performed on these variables to examine them both collectively and individually. Because no standard transformation uniformly corrected both the heteroscedasticity and non-normality of the ANOVA model residuals, the variables were rank transformed. The above analyses were thus run on both the untransformed and rank-transformed variables. Multivariate analysis of neither the untransformed nor the rank transformed data indicated any multivariate effect of fog oil smoke concentration. The univariate results shown in Table 12 were at least marginally significant.

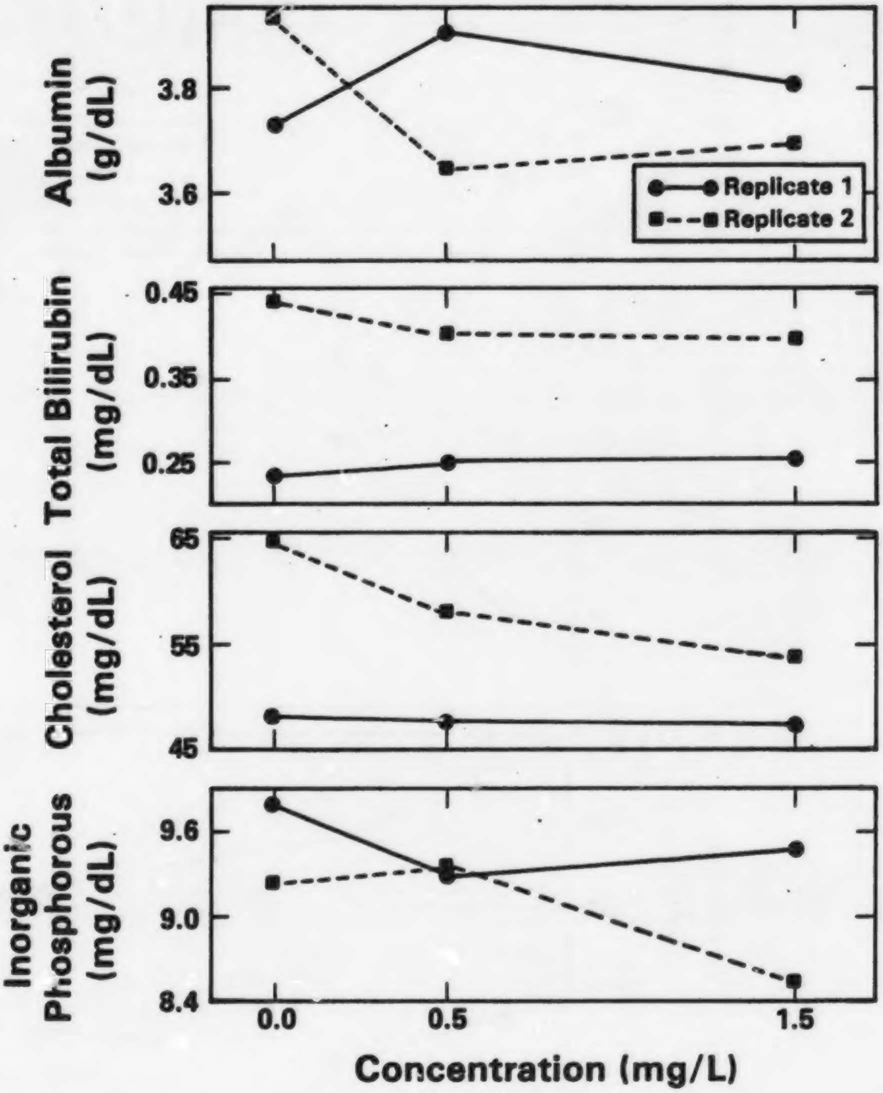

Figure 27. Effects of 4-wk fog oil smoke exposure on serum albumin, total bilirubin, cholesterol, and inorganic phosphorus by replicate.

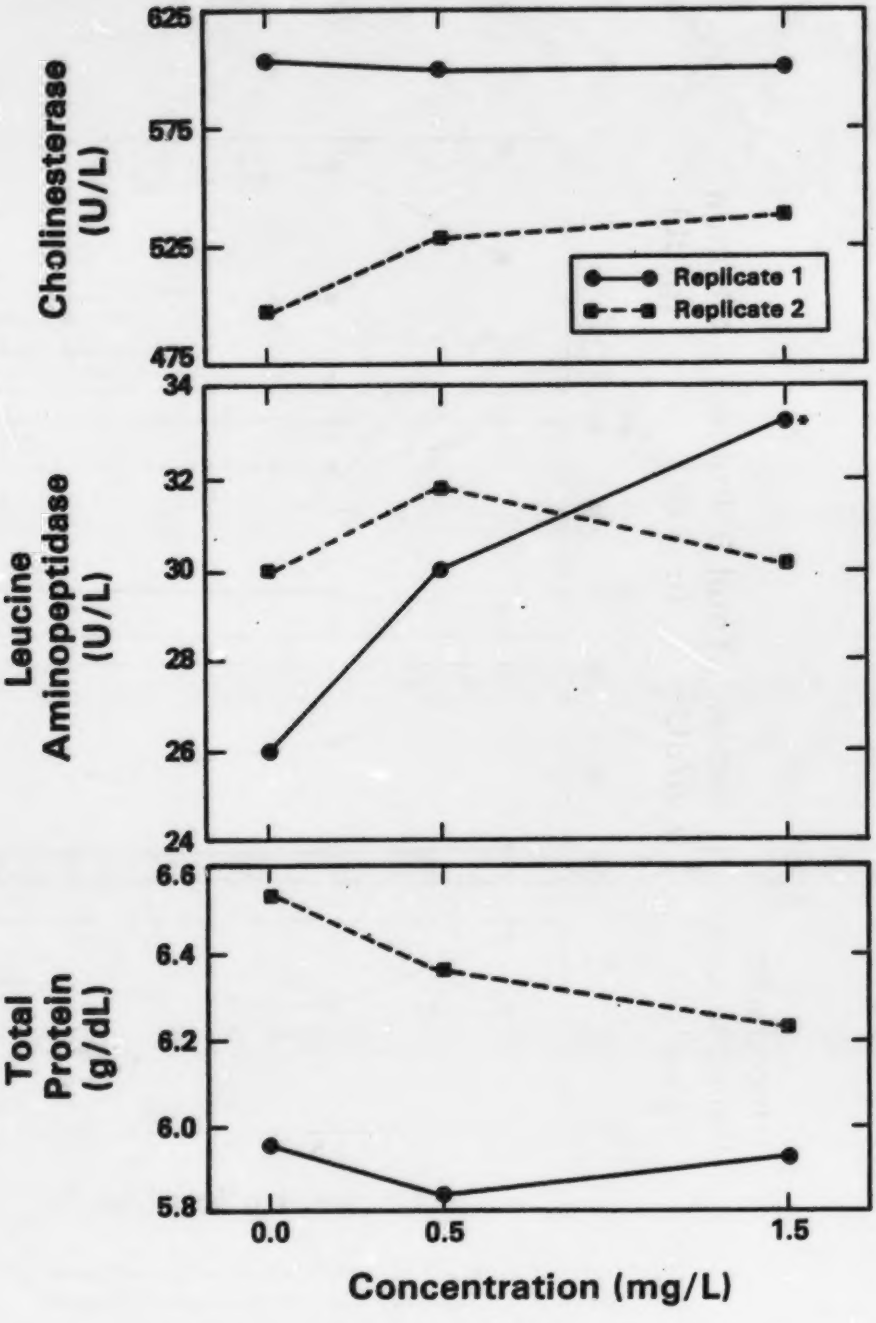

Figure 28. Effects of 4-wk fog oil smoke exposure on serum cholinesterase, leucine aminopeptidase, and total protein by replicate. *Significantly different from control ($p < 0.05$).

TABLE 12. UNIVARIATE RESULTS OF HEMATOLOGY PARAMETERS

Variable	Contrast	Exposure	p	Critical p[a]
MCV	Air vs. 0.5 mg/L	4 days/wk-70 min	0.018	0.006
MCV	Air vs. 1.5 mg/L	4 days/wk-70 min	0.010	0.006
Hemoglobin	Air vs. 0.5 mg/L	4 days/wk-70 min	0.006	0.006

[a] $p \leq 0.05 \div 8$.

A consistent trend toward significance of a particular exposure regimen over several end points did not emerge, nor were there effects in the worst case exposures (highest concentration for the longest and most frequent exposure). No notable effects of fog oil smoke exposure appeared on hematology end points, at least with the sample sizes used here. However, when hematology parameters measured in replicates 1 and 2 were analyzed together by a multivariate ANOVA model some effects were noted. There was no indication of a multivariate interaction ($p = 0.64$, Wilks' criterion) or an overall fog oil smoke concentration effect ($p = 0.18$, Wilks' criterion). The univariate ANOVA for MCV indicated a highly significant main effects test ($p = 0.0054$) due to exposure concentration (Figure 29). Changes in the RBC were marginally significant, given the number of responses examined. Thus, although the volume of red blood cells was significantly decreased with increasing concentrations of fog oil smoke ($p = 0.05$ at the 1.5-mg/L level), the RBC appeared to increase with fog oil smoke concentration. The other parameters were not affected by fog oil smoke exposure (Figure 30).

Xenobiotic Metabolism

Pentobarbital-Induced Sleeping Time

The parameters measured by this assay, time to loss of righting reflex after injection of sodium pentobarbital and elapsed sleeping time, were examined using both multivariate and univariate ANOVA methods. When the saturated three-way multivariate ANOVA was fit to the vector response comprised of these two times, there was a significant effect on the joint response due to exposure time ($p = 0.043$) and a marginally significant interaction ($p = 0.09$) of exposure frequency and concentration. To investigate these effects further, the univariate three-way ANOVA of each response variable was examined. The analysis of loss of righting reflex times mimicked the multivariate findings with the significant effect ($p = 0.0279$) due to exposure time and a marginally significant ($p = 0.0833$) exposure frequency by concentration interaction (Figure 31). Least square estimates of mean times to loss of righting reflex were 11.07 and 9.65 min for the 70-min and 3.5-hr exposure groups, respectively. Animals exposed 4 days/wk showed a decreasing mean number of minutes to loss of righting reflex with increasing fog oil smoke concentration. However, animals exposed 2 days/wk showed a

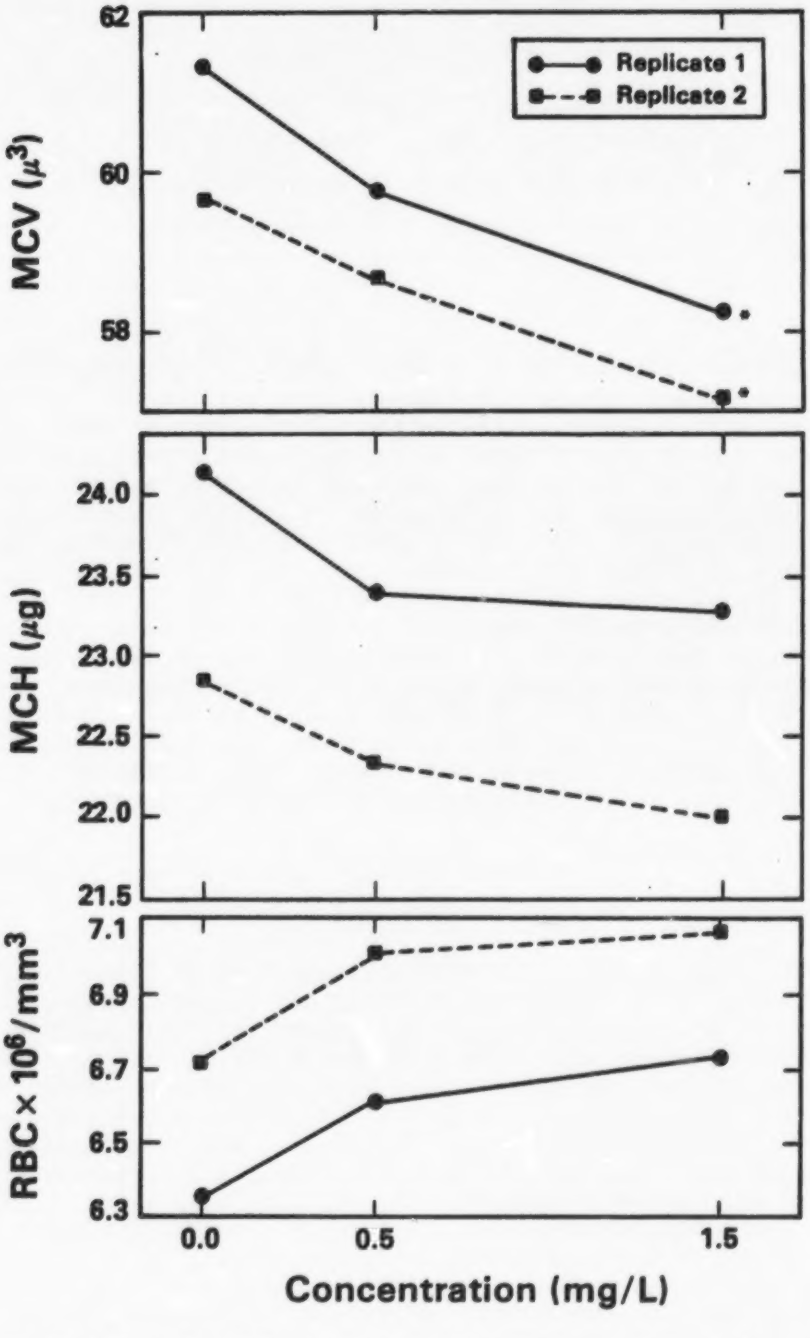

Figure 29. Effect of 4-wk fog oil smoke exposure on mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and red blood cell count (RBC) by replicate. *Significantly different from control ($p < 0.05$).

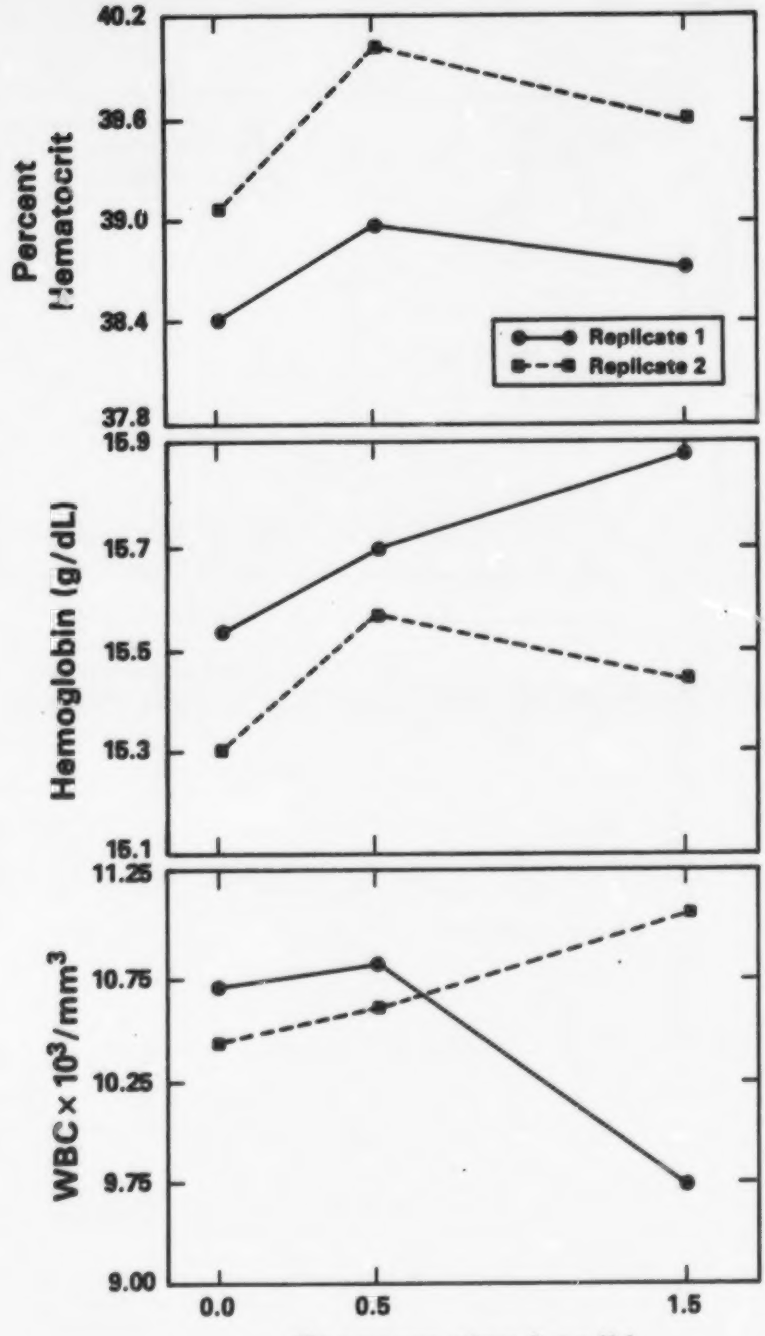

Figure 30. Effect of 4-wk fog oil smoke exposure on percent hematocrit, hemoglobin, and white blood cell count (WBC) by replicate.

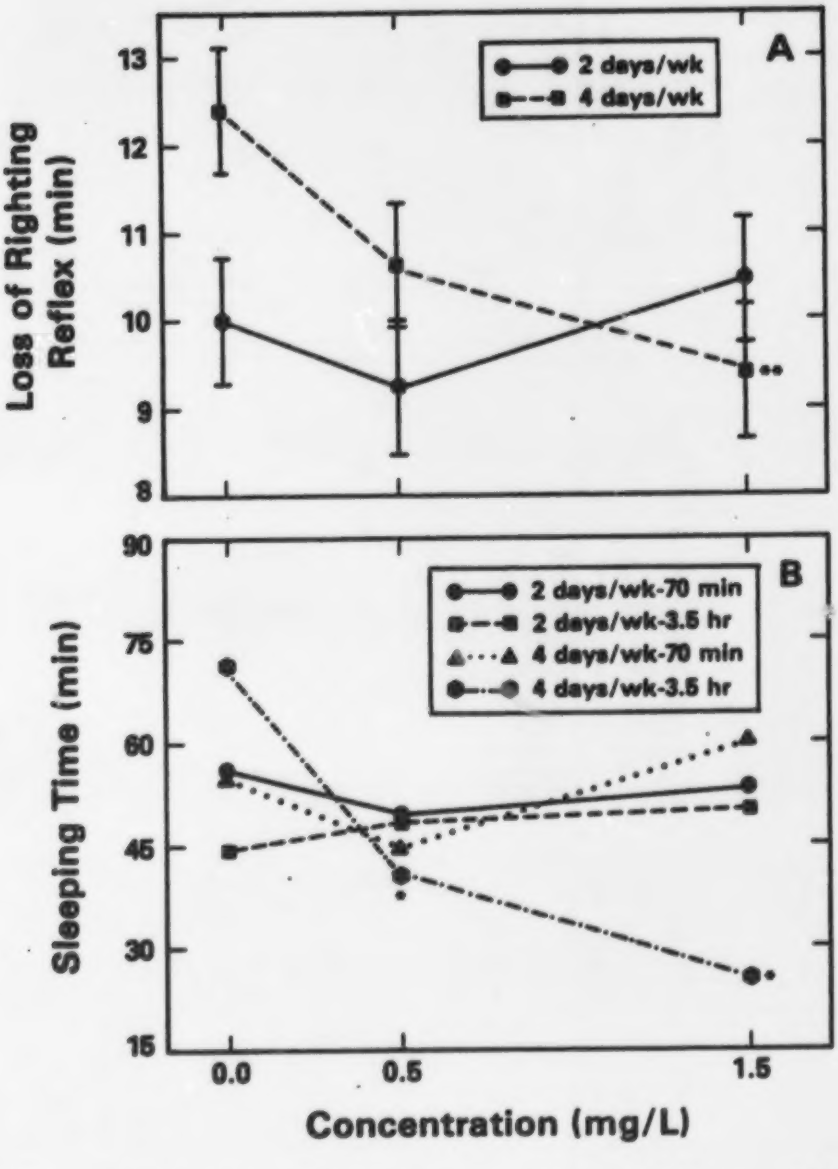

Figure 31. Effect of 4-wk fog oil smoke exposure on pentobarbital metabolism. A. Loss of righting reflex; B. Sleeping time. Error bars represent the standard error of the mean. *Significantly different from control ($p < 0.05$). **Significantly different from control ($p < 0.01$).

decline in loss of righting reflex time at 0.5 mg/L of fog oil smoke but showed an increase at 1.5 mg/L. Using subtests of the differences in least square means for the animals exposed 4 days/wk, the response at 1.5 mg/L (9.4 min) was markedly different ($p = 0.006$) from the control response. For the animals exposed 2 days/wk, there were no significant differences between controls and fog oil smoke exposed groups.

With respect to pentobarbital-induced sleeping time, there were no significant effects due to concentration, frequency of exposure, or exposure duration. The least square means from the analysis reflected a trend in the expected direction. Mean sleeping time for animals exposed 2 days/wk was 50.2 min and for animals exposed 4 days/wk was 49.1 min. For rats exposed 70 min and 3.5 hr on each occasion, sleeping times were 52.7 and 46.7 min, respectively. At 0.0, 0.5, and 1.5 mg/L, sleeping times were 56.1, 45.6, and 47.4 min, respectively. Figure 31 plots mean sleeping times over fog oil smoke concentration for each combination of exposure frequency and duration. Only in animals exposed 3.5 hr/day for 4 days/wk was there any indication of the expected concentration response; the concentration response in the other three groups was generally flat. When we analyzed only the 3.5-hr exposure group, we saw a significant ($p = 0.02$) effect due to fog oil smoke concentration. Although this test was biased because it was performed after the fact, it did indicate that sleeping time was more than negligibly affected by fog oil smoke concentration.

Figure 32 shows a comparison of replicates 1, 2, and 3 for the 3.5-hr/day, 4-day/wk for 4-wk exposures. Considering the third replicate data separately, sleeping time increased slightly with increasing fog oil smoke concentration. This contrasted with the previous replicates in which a monotonic decrease in sleeping time was seen with increasing concentration. The result was a highly significant ($p < 0.01$) interaction between REP and CONC. The data were then examined taking two replicates at a time. Only when the second and third replicate data were combined was the interaction not statistically significant. There was no difference, however, between sleeping times of animals from the different fog oil smoke concentrations when pooled over these two replicates.

Zoxazolamine-Induced Paralysis Time

This end point was measured only for animals exposed 4 days/wk as explained earlier. Two measurements were taken, the time from injection to loss of righting reflex and the time the animal remained paralyzed. Because the response is multidimensional, both multivariate and univariate methods were used to analyze the data. Logarithms of both measures were taken to improve homogeneity of variance and the normal distribution of residual assumptions. A saturated two-way multivariate ANOVA model was fit to the response vector comprised of both loss of righting reflex and paralysis times. This joint response was significantly affected by both exposure concentration ($p = 0.0001$) and duration ($p = 0.0004$).

The univariate analyses indicated that most of the effects seen in the multivariate ANOVA arose with respect to paralysis time and not loss of

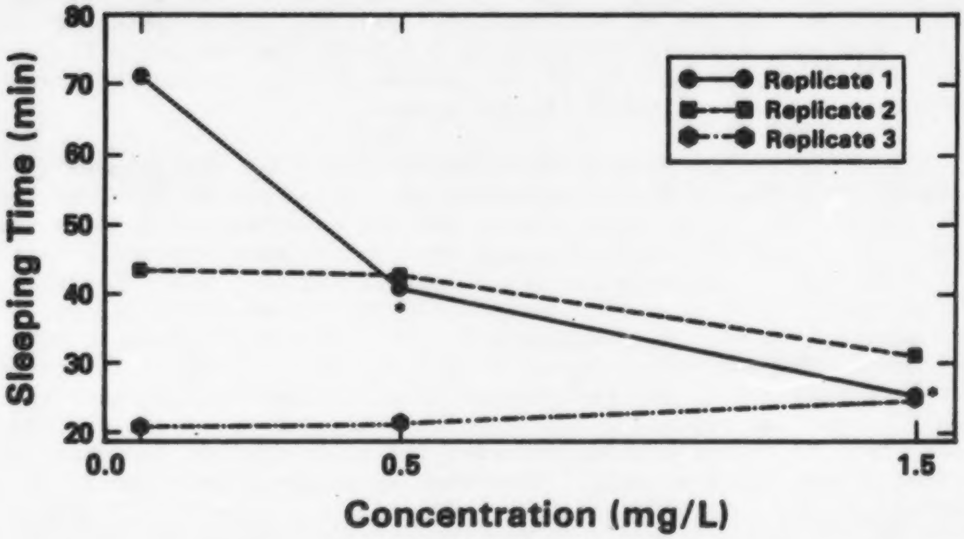

Figure 32. Effect of 4-wk fog oil smoke exposure on pentobarbital-induced sleeping time by replicate. *Significantly different from control ($p < 0.05$).

righting reflex. The ANOVA of the latter indicated no significant main or interactive effects from concentration or exposure duration. Figure 33 depicts mean loss of righting reflex with increasing fog oil smoke concentration for each exposure duration. The geometric mean loss of righting reflex was 4.42 min in controls, and was 4.45 and 4.99 min in the 0.5- and 1.5-mg/L concentration groups, respectively. With respect to exposure duration, mean times to loss of righting reflex were 4.42 and 4.81 min for the 70-min and 3.5-hr exposure groups, respectively.

The univariate ANOVA of paralysis time indicated highly significant effects due to both exposure concentration of fog oil smoke ($p = 0.0001$) and duration ($p = 0.0001$; Figure 33). Least squares geometric means were 228, 108, and 87 min for the air control, 0.5-mg/L, and 1.5-mg/L exposure groups, respectively. For the 70-min and 3.5-hr exposure groups, geometric mean responses were 162 and 103 min, respectively. From Figure 33, we can clearly see there is no evidence of an exposure concentration by duration interaction. Thus, we can reasonably assume that, for paralysis time, these two factors are additive.

Figure 34 shows a comparison of the results from replicates 1 and 2. In both replicates, paralysis time was significantly depressed after exposure to both 0.5 and 1.5 mg/L of fog oil smoke in a concentration-dependent manner. Zoxazolamine induction time was not affected by fog oil smoke exposure (data not shown).

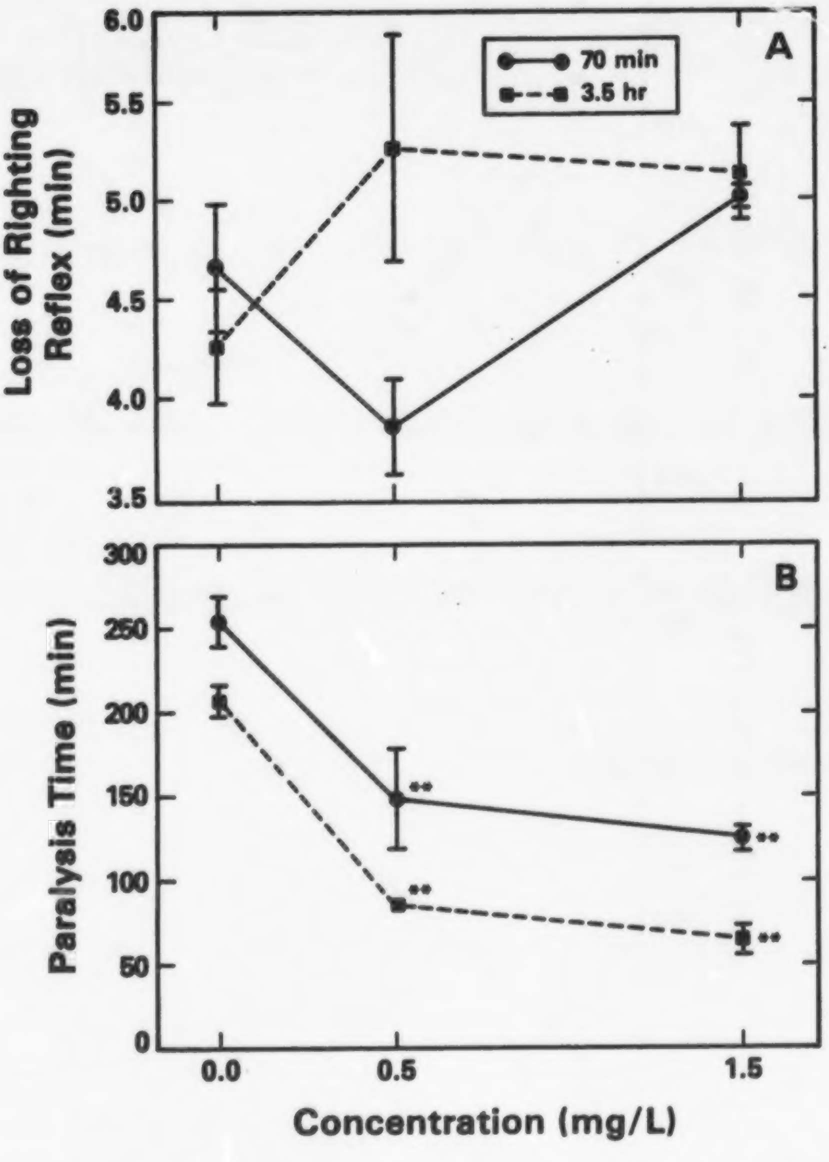

Figure 33. Effect of 4-wk fog oil smoke exposure on zoxazolamine metabolism. A. Loss of righting reflex; B. Paralysis time. Error bars represent the standard error of the mean. **Significantly different from control ($p < 0.01$).

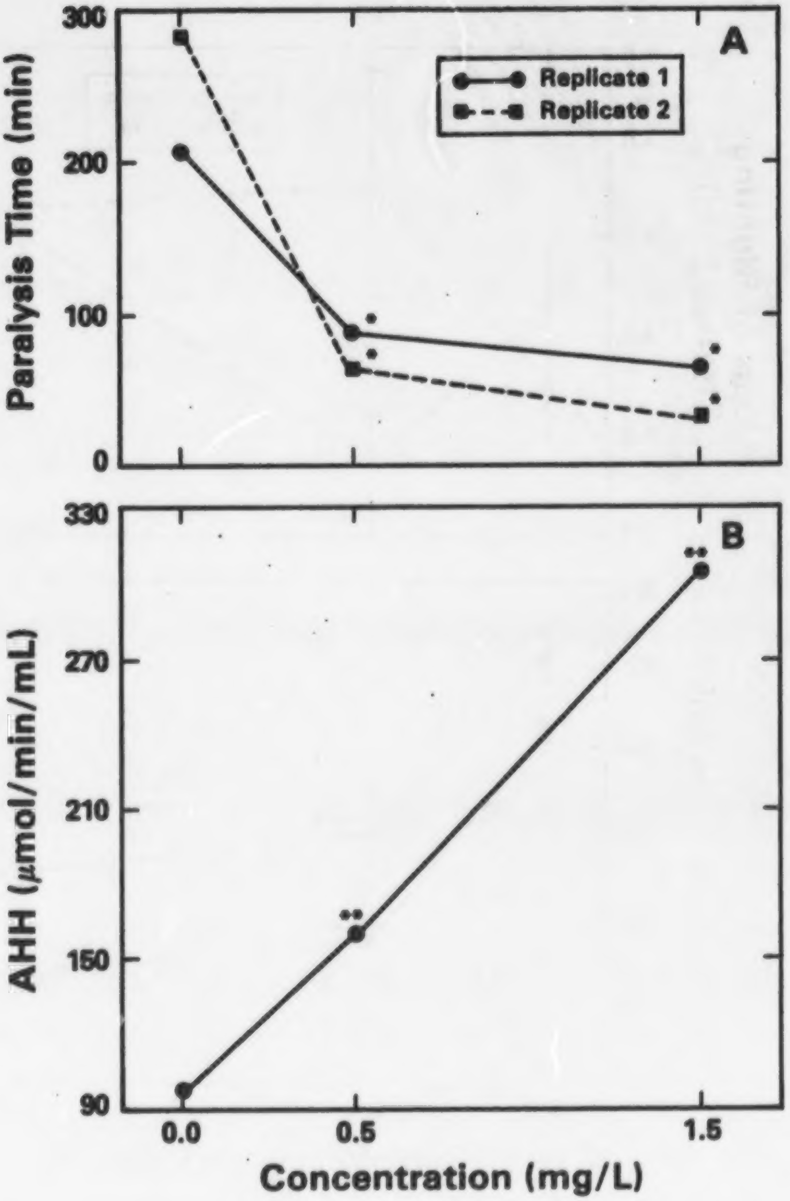

Figure 34. Effect of 4-wk fog oil smoke exposure on xenobiotic metabolism by replicate. A. Paralysis time; B. AHH activity. *Significantly different from control ($p < 0.05$). **Significantly different from control ($p < 0.01$).

Enzyme Activity

This end point was not assayed during the previous 4-wk exposures. Because there was only one replicate, a one-way ANOVA by concentration was run against each response. In the case of a significant ($p < 0.01$) main effect of concentration, the Williams' test for lowest effective concentration was applied. AHH showed concentration-related effects ($p < 0.001$, Figure 34). The Williams' test indicated that the AHH mean at 0.5 and 1.5 mg/L was significantly different from that of controls.

Whole-Body versus Nose-Only Exposures (Zoxazolamine-Induced Paralysis Time)

The comparison study of whole-body to nose-only exposure using zoxazolamine-induced paralysis time as an end point was analyzed. Analysis consisted of a two-way ANOVA on the primary parameter of interest, zoxazolamine-induced paralysis time. The two factors were the method of exposure, whole-body (WB) or nose-only (NS), and the fog oil smoke concentration of 1.5 mg/L or filtered air.

ANOVA indicated no sign of an interactive or main effect on paralysis time due to the method of exposure. Either method or pooling over methods indicated a highly significant effect due to fog oil smoke exposure at 1.5 mg/L (Figure 35).

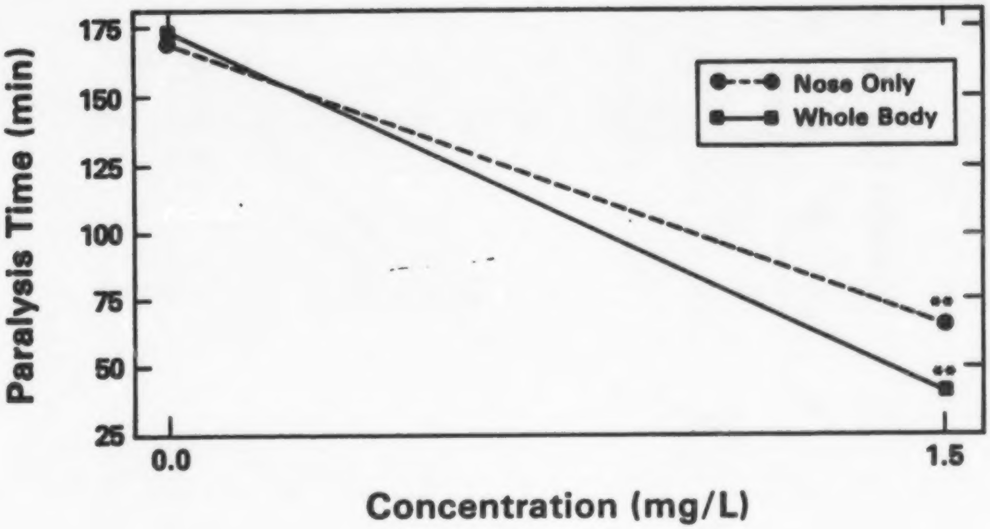

Figure 35. Comparison of whole-body and nose-only exposure methods on paralysis time. **Significantly different from control ($p < 0.01$).

Immunology

Natural killer (NK) cell activity was determined at four effector-to-target cell ratios ranging at twofold intervals from 1 to 25 to 1 to 200. The multivariate ANOVA showed only an effect due to concentration ($p = 0.05$). Figure 36 shows that this was due to an increase in cytotoxicity in the fog oil smoke-exposed groups. Figure 37 shows a graph of NK cell activity for the different exposure groups. NK cell activity increased in fog oil smoke-treated animals exposed 4 days/wk for either 70 min or 3.5 hr, and 2 days/wk for 3.5 hr. However, post hoc t tests showed that the only results approaching significance were at the 1:200 and 1:50 ratios. For the 1:200 ratio, the difference between the control and the 1.5-mg/L group means for the 2 days/wk-3.5 hr exposure yielded a significance of $p = 0.02$. The same contrast for the 4-day/wk group for the same duration time did not approach significance. For the 1:50 assay, the difference between the control and the 1.5-mg/L group means for 4 days/wk-3.5 hr/day yielded a significance of $p = 0.03$.

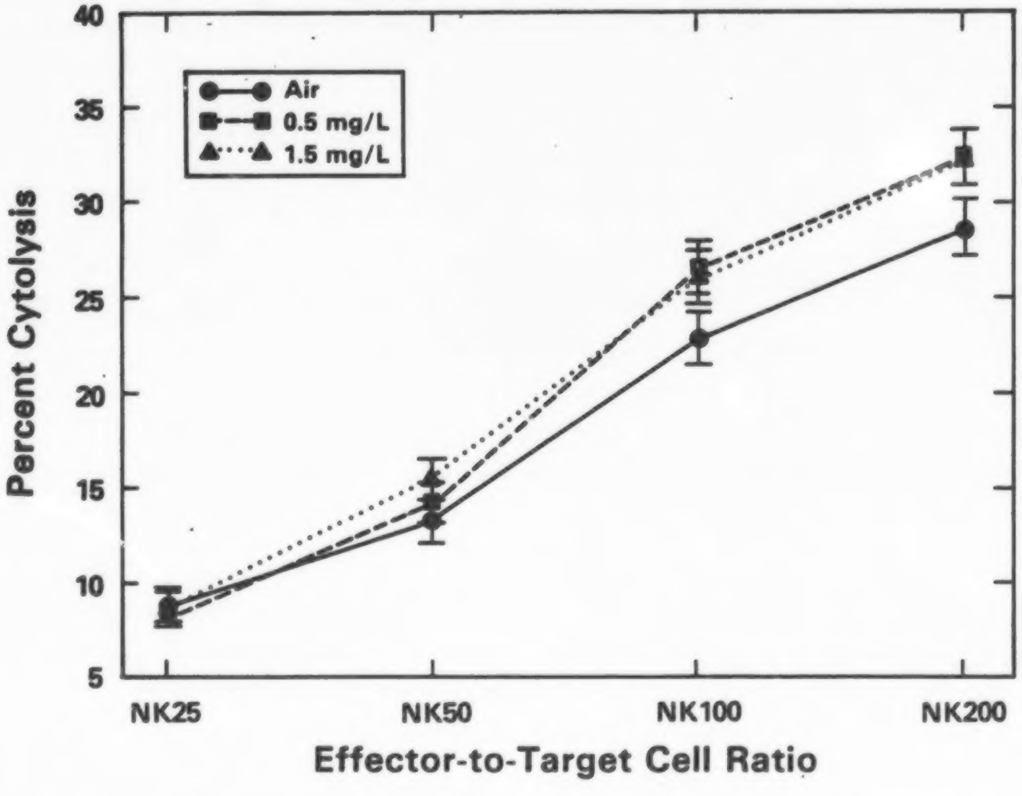

Figure 36. Effect of 4-wk fog oil smoke exposure on NK cell cytolytic activity. Error bars represent the standard error of the mean.

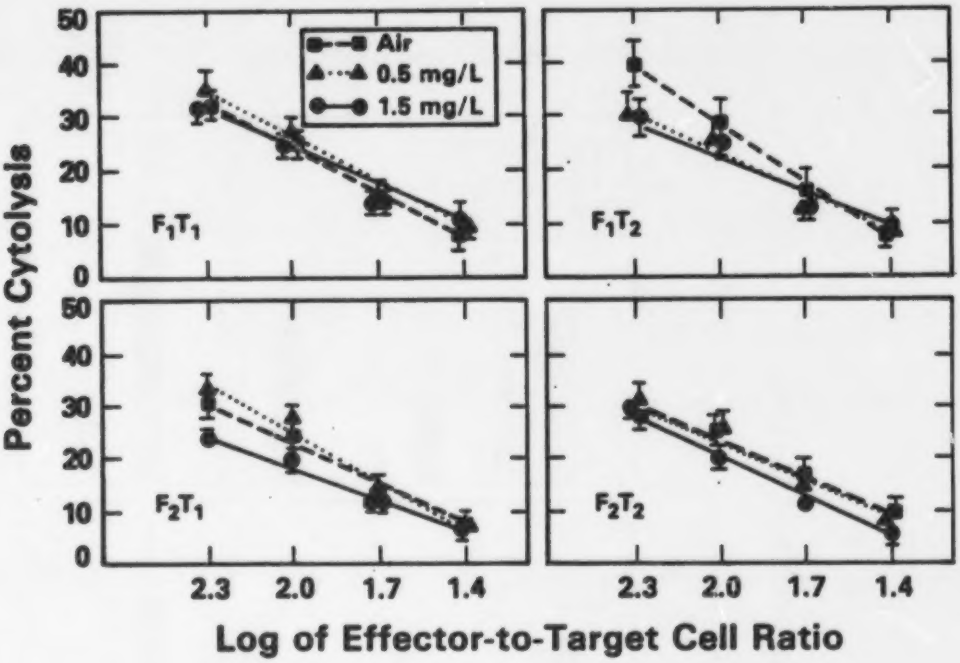

Figure 37. NK cell cytolytic activity for the different exposure groups expressed as log of effector-to-target cell ratio. F_1 = 2 days/wk; F_2 = 4 days/wk; T_1 = 70 min/day; T_2 = 3.5 hrs; 1.4 = NK25; 1.7 = NK50; 2.0 = NK100; 2.3 = NK200. Error bars represent the standard error of the mean.

Responses to mitogens are expressed as counts obtained for the individual mitogen treatments less background counts obtained from cells exposed only to media. For statistical analysis, square root-transformed values were used to improve variance homogeneity and normality of residuals. Multivariate ANOVA of transformed spleen cell data indicated a significant frequency effect ($p = 0.0005$) but no effects due to concentration or time. A similar analysis of peripheral blood cells also showed frequency as the only significant effect ($p = 0.0045$). Univariate ANOVA was also performed for each mitogen tested in spleen and for each mitogen tested in peripheral blood. Eight post hoc t tests comparing the control versus each fog oil smoke exposure group mean for each frequency-exposure-time combination were examined to determine for which, if any, a significant concentration-related effect could be seen. For both spleen (Figure 38) and peripheral blood (Figure 39), only the differences between the control mean and the 1.5-mg/L mean in the longest exposure and the highest frequency group appeared to approach significance. For this group, the spleen two-sided p values for the mitogens PHA, Con A, and PWM were 0.04, 0.03, and 0.06, respectively. For peripheral blood, p values for the same mitogens were 0.03, 0.07, and 0.05; however, if these values were adjusted for the number of contrasts examined, these results would not reach significance. Also, in the second replicate, this trend toward decreasing response to mitogens with increasing fog oil smoke concentrations for both spleen and peripheral blood culture did not occur (Figures 40 and 41).

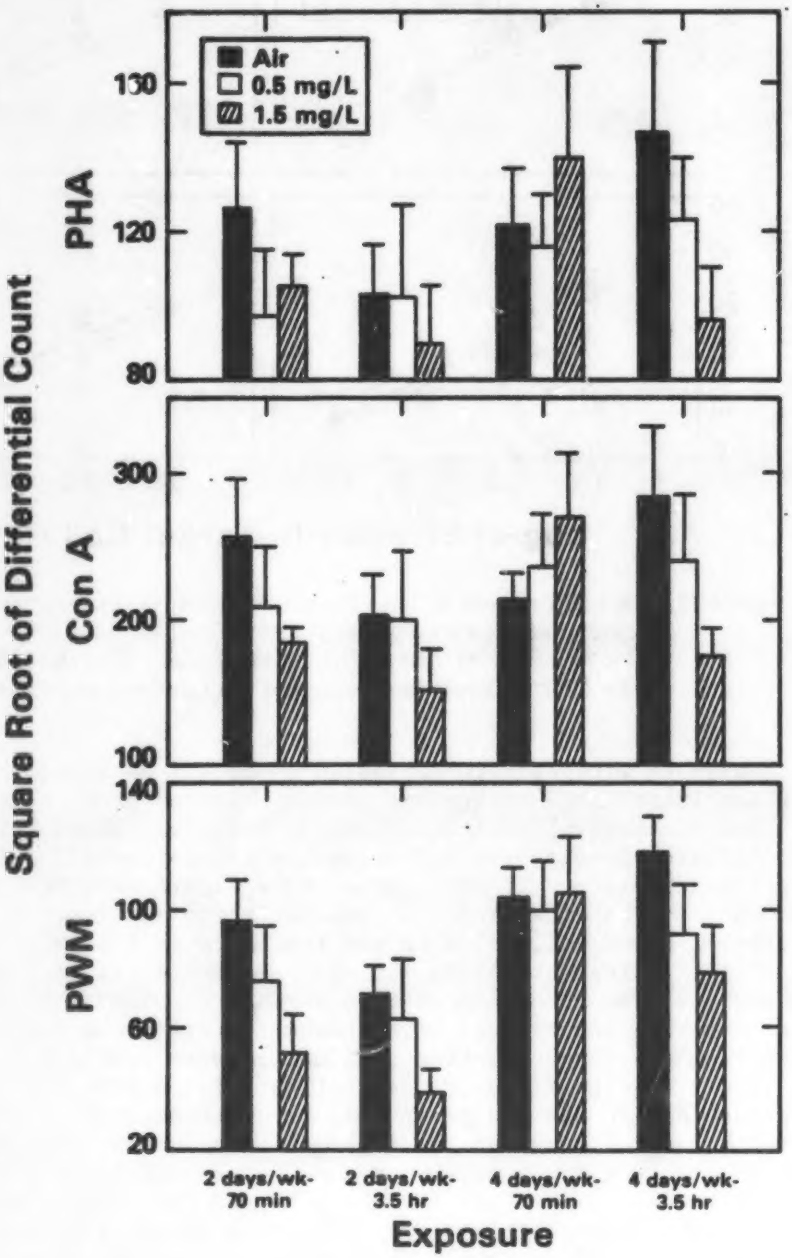

Figure 38. Effect of 4-wk exposure on response of spleen cells to PHA, Con A, and PWM. Error bars represent the standard error of the mean.

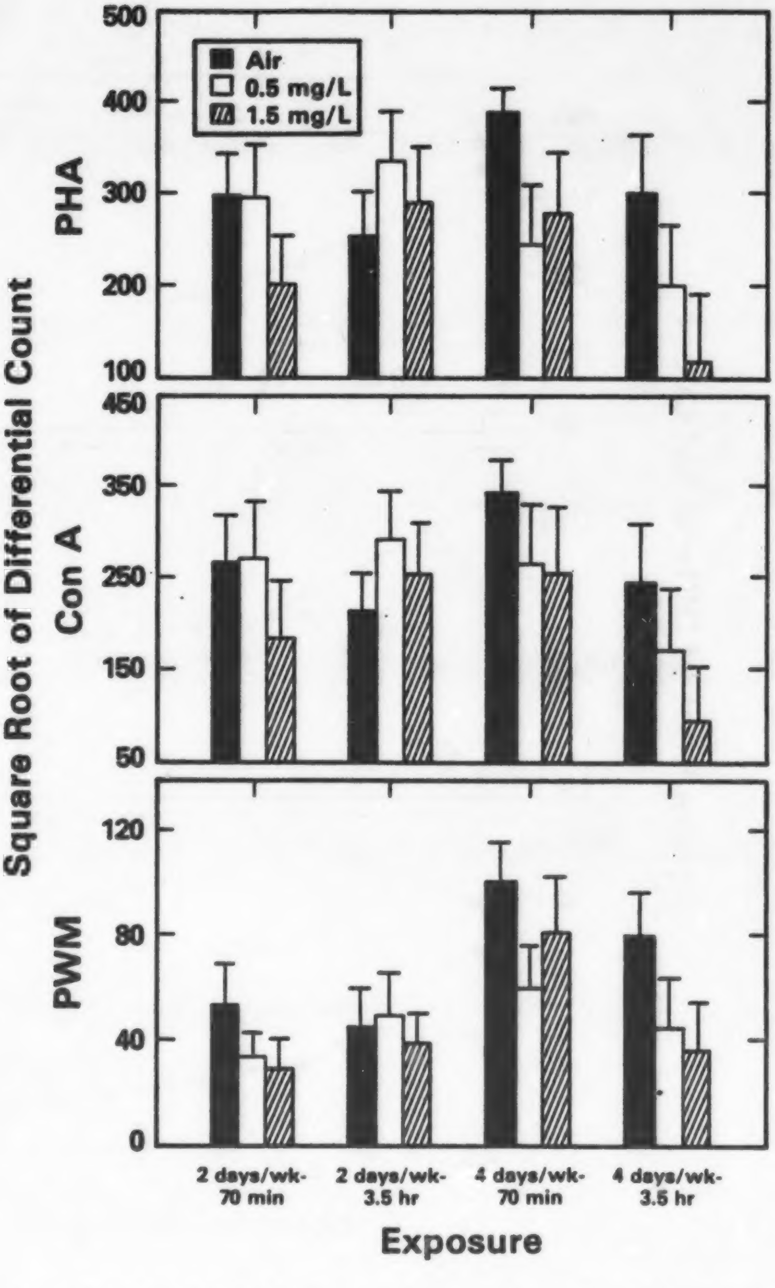

Figure 39. Effect of 4-wk exposure on response of peripheral blood to PHA, Con A, and PWM. Error bars represent the standard error of the mean.

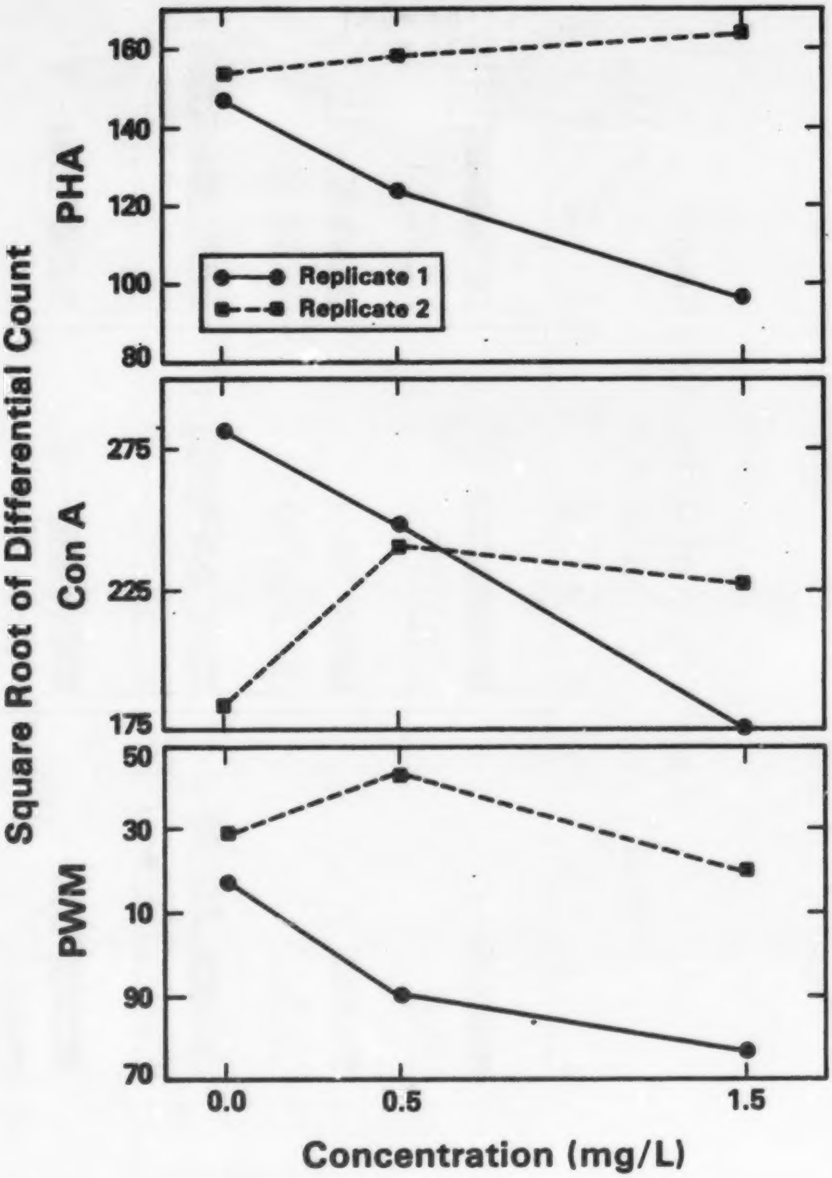

Figure 40. Response of spleen cells to PHA, Con A, and PWM by replicate after 4 wk of exposure.

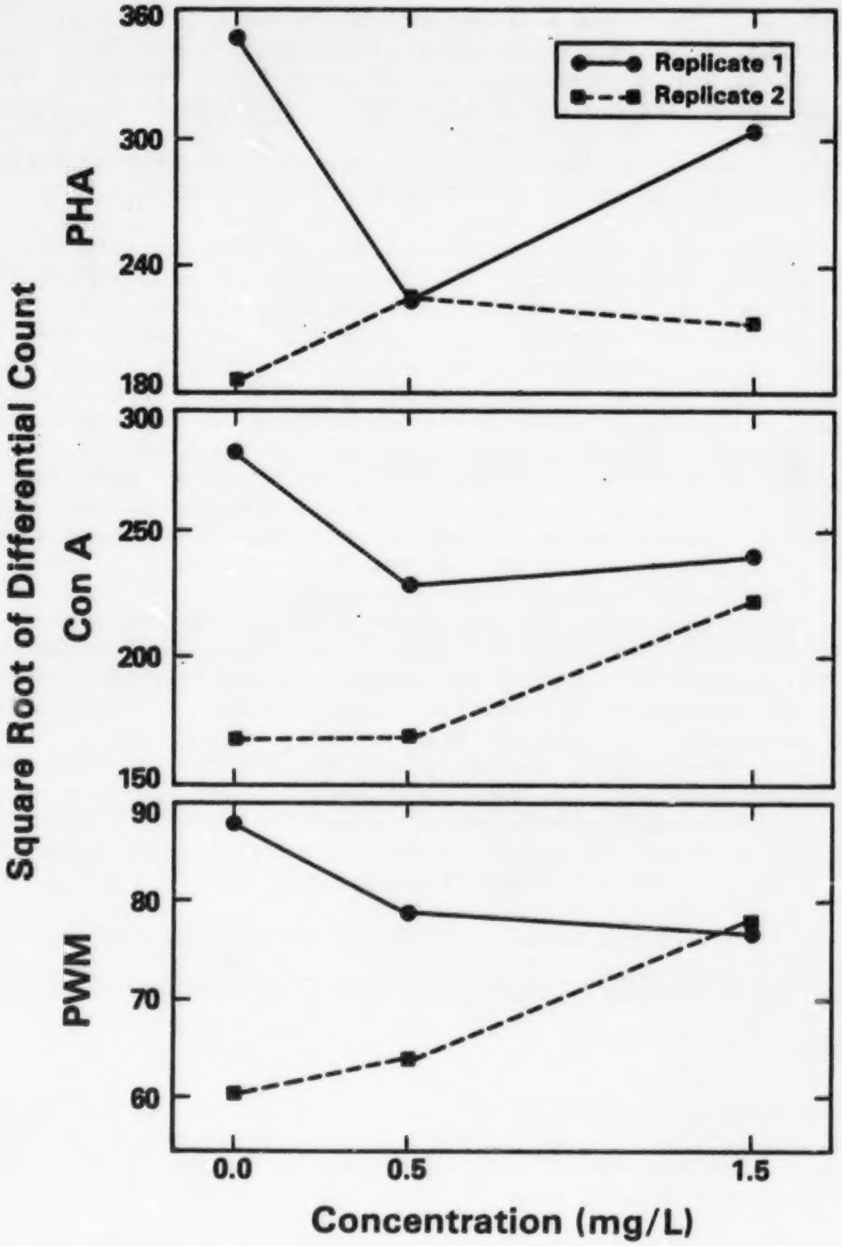

Figure 41. Response of peripheral blood to PHA, Con A, and PWM by replicate after 4 wk of exposure.

Square roots of differential counts were analyzed for responses of splenic and peripheral lymphocytes to mitogens. Multivariate ANOVA of these variables by replicate and concentration indicated a weak interaction ($p = 0.091$); however, there was no evidence of a multivariate effect due to concentration ($p = 0.66$). There was a strong effect due to replicate ($p = 0.002$). None of the univariate ANOVAs indicated either a significant ($p \leq 0.1$) interactive or main effect due to concentration. None of these variables indicated a significant concentration-related effect when analyzing only the second replicate data. If there were effects due to these fog oil smoke concentrations with respect to the immunology parameters analyzed here, we did not have enough power to detect them.

A third replicate was conducted for NK cell activity because the results of the first two replicates were inconclusive. Four effector-to-target cell ratios were tested at twofold intervals ranging from 1:200 to 1:25. Results for each effector-to-target cell ratio were analyzed separately by two-way ANOVA. For all replicates combined, no main effect of fog oil smoke concentration was detectable for NK50, NK100, or NK200 (Figure 42). For NK25, an interaction ($p = 0.054$) between replicate and fog oil smoke concentration was indicated. Apparently this was because the second replicate (which was comprised of only three animals per group) differed markedly from the first and third replicate, each of which showed enhanced NK cell activity with increasing fog oil smoke concentration. Looking at these analyses two replicates at a time, it was evident that replicates 1 and 3 reflected the most consistent response. This can be seen from the interaction between replicate and exposure concentration for each of four ratios. These two replicates also indicated main effects due to fog oil smoke exposure not only for NK25, but also for NK50 and NK100. Pooled over these two replicates, tests of differences between least square means indicated that for NK25, the mean percentage of lysis was significantly higher than control response at both 0.5 and 1.5 mg/L; for NK50, only the mean at 1.5 mg/L was significantly higher than control; and for NK100, results were the same as for NK25. Thus, if the second replicate (3 rat) is ignored, the data showed enhanced NK cell activity at three of the four effector-to-target cell ratios in rats exposed to 1.5 mg/L of fog oil smoke (Figure 43).

Table 13 shows a summary of the subacute range-finding study results.

Phase III - Subchronic Studies

Part A

In this study, male rats were exposed for 13 wk, 3.5 hr/day, 4 days/wk, to filtered air, or 0.5- or 1.5-mg/L fog oil smoke concentrations. Half the animals were assessed the day after the last exposure (nonrecovery) and half were assessed after a 4-wk recovery period (recovery).

Histopathology

Hematoxylin- and eosin-stained histologic sections were prepared and evaluated histopathologically from the following tissues: eyes, heart, skin (mid-dorsal region), larynx, peribronchial lymph node, trachea, right

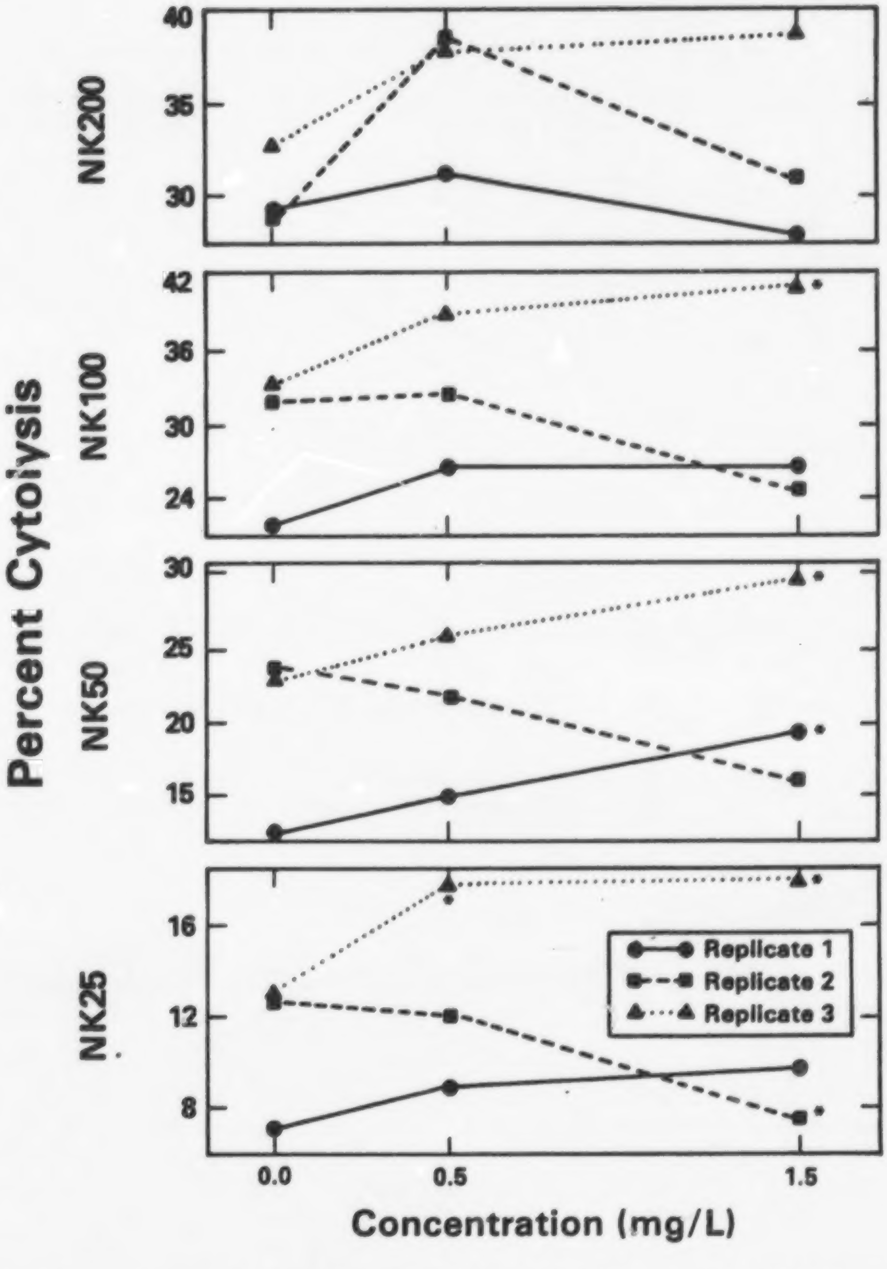

Figure 42. Response of NK cell cytolytic activity by replicate after 4 wk of exposure. *Significantly different from control ($p < 0.05$).

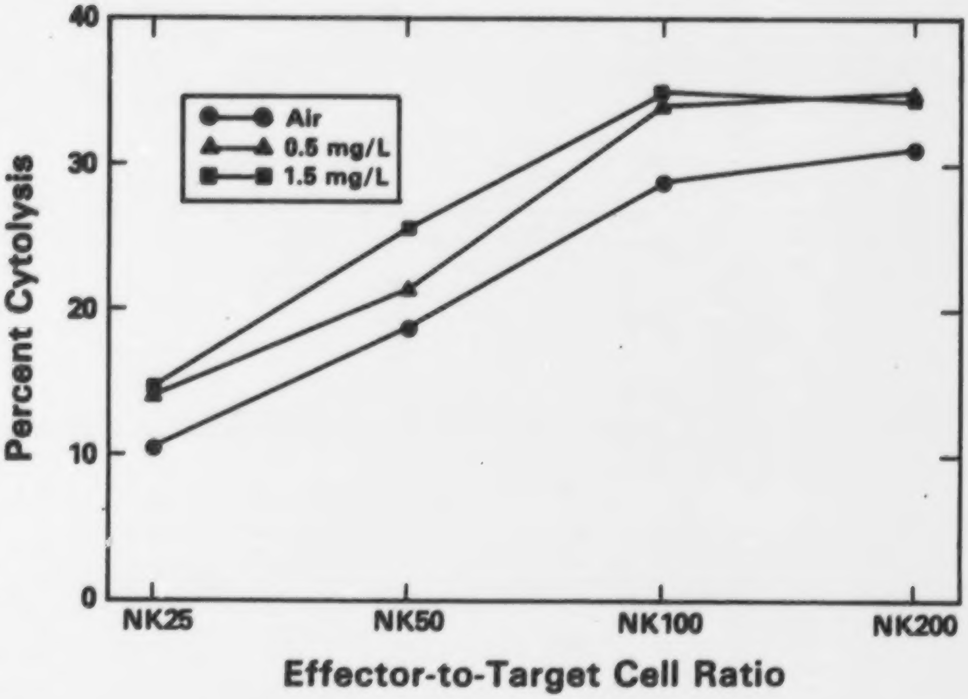

Figure 43. Effects of 4-wk fog oil smoke exposure on NK cell cytolytic activity expressed as effector-to-target cell ratio.

diaphragmatic lobe of the lung, left lobe of the lung, kidneys, liver (left lobe), stomach, duodenum, testes, epididymides, and three sections of the nasal cavity.

Treatment-related microscopic changes were observed in the lungs and the peribronchial lymph nodes of male rats in both concentration groups following the 13-wk exposure to fog oil smoke. Similar changes of decreased severity were present in the lung and peribronchial lymph nodes in male rats following the recovery period.

TABLE 13. SUMMARY OF PHASE II - SUBACUTE RANGE-FINDING STUDIES

Parameter	Effect
Histopathology	Dose-related ↑ in alveolar macrophages Pneumonitis in 4 of 6 males exposed at 1.5 mg/L
Pulmonary physiology	↑ EEV, lung wet and dry weight
Lavage fluid protein	↑ at 1.5 mg/L No male/female difference
Pulmonary cells	↑ in PMNs and total cells at 1.5 mg/L
Behavioral response	NS[a]
Clinical chemistry	NS[a]
Hematology	NS[a]
Pentobarbital-induced sleeping time	NS[a]
Zoxazolamine-induced paralysis time	Decreased at 0.5 and 1.5 mg/L
AHH activity	Increased at 0.5 and 1.5 mg/L
Immunology	Possible enhancement of NK cells

a. NS = no significant effects.

In male rats exposed for 13 wk to fog oil smoke, a diffuse accumulation of macrophages was present in the pulmonary alveoli. Although the distribution of macrophages involved all regions of the pulmonary parenchyma, the number of alveolar macrophages in the alveoli was greater near the terminal bronchioles. The degree of severity was concentration related with moderate to moderately severe involvement in the 1.5-mg/L exposure group, and minimal to slight involvement in the 0.5-mg/L exposure group. Similar changes were present following recovery; however, the degree of involvement was slightly decreased. Only a moderate number of alveolar macrophages were present in the lungs of male rats in the 1.5-mg/L recovery group. There was very little difference between the appearance of the lung after 13-wk exposure or following the recovery period at 0.5 mg/L with minimal to slight degrees of alveolar macrophages being present in both groups. Other changes observed in the lungs that appeared to be associated with fog oil smoke exposure included focal hemorrhage in 3 of 10 rats in the 1.5-mg/L exposure group following a 13-wk exposure. Multifocal granulomatous pneumonia was observed in 1 of 10 rats following the 13-wk exposure and in 2 of 10 rats following recovery in the 1.5-mg/L-exposed group. Other changes that were observed within the lung were considered to be incidental and most frequently included peribronchial and perivascular lymphocytic infiltrates.

Multiple foci of macrophage accumulation were present in the cortical and medullary sinusoids of the peribronchial lymph nodes of male rats in all exposed groups but not in control groups. Lymphoid hyperplasia was also observed in the cortex of the peribronchial lymph nodes of many of the exposed rats. The incidence and the severity of these changes were both concentration related. However, little difference was present between the incidence or severity at either exposure level when the findings at 13 wk were compared with those following the recovery period.

At necropsy, the mandibular lymph nodes of several of the control and exposed rats were described as slightly enlarged and red. Microscopically, these lymph nodes had varying degrees of lymphoid hyperplasia and congestion of the cortical and medullary sinusoids. Although the incidence of these findings was slightly greater in exposed groups than in the control groups, the relationship to exposure is not clear. Only mandibular lymph nodes described as abnormal at necropsy were examined histopathologically; therefore, this tissue was not examined from all rats in the study.

No treatment-related changes were present in other tissues examined in this phase of the study. A few incidental lesions such as chronic myocarditis, tubular regeneration in the kidney, and seminiferous tubular atrophy in the testes were occasionally observed. These changes were considered to be within normal limits for male rats of this age and strain.

The terminal body, brain, lung, liver, kidney, and testicle weights were recorded at necropsy. For other end points, the pathology data were analyzed both separately by type of recovery and by combining recovery and nonrecovery animals. Total body weight and the weights of each organ are plotted in Figure 44, and the ratios of these organs to total body weight are shown in Figure 45. Only these ratios or transformations of them were analyzed within a multivariate ANOVA framework. All ratio data was log-transformed to alleviate heteroscedasticity problems.

Multivariate ANOVA of the nonrecovery data of the logs of the tissues-to-body weight ratios indicated a significant effect on the joint response ($p = 0.0001$, Wilks' criterion) due to fog oil smoke concentration. Univariate ANOVAs indicated concentration-related effects for the lung ($p = 0.001$) and liver ($p = 0.04$) to body weight ratios. The Williams' test, assuming a monotonically increasing or decreasing trend with increasing concentration, indicated that even at 0.5 mg/L, lung-to-body weight ratios were significantly different from those of controls. Brain, liver, and kidney weights as a percentage of total body weight also increased with concentration but not significantly.

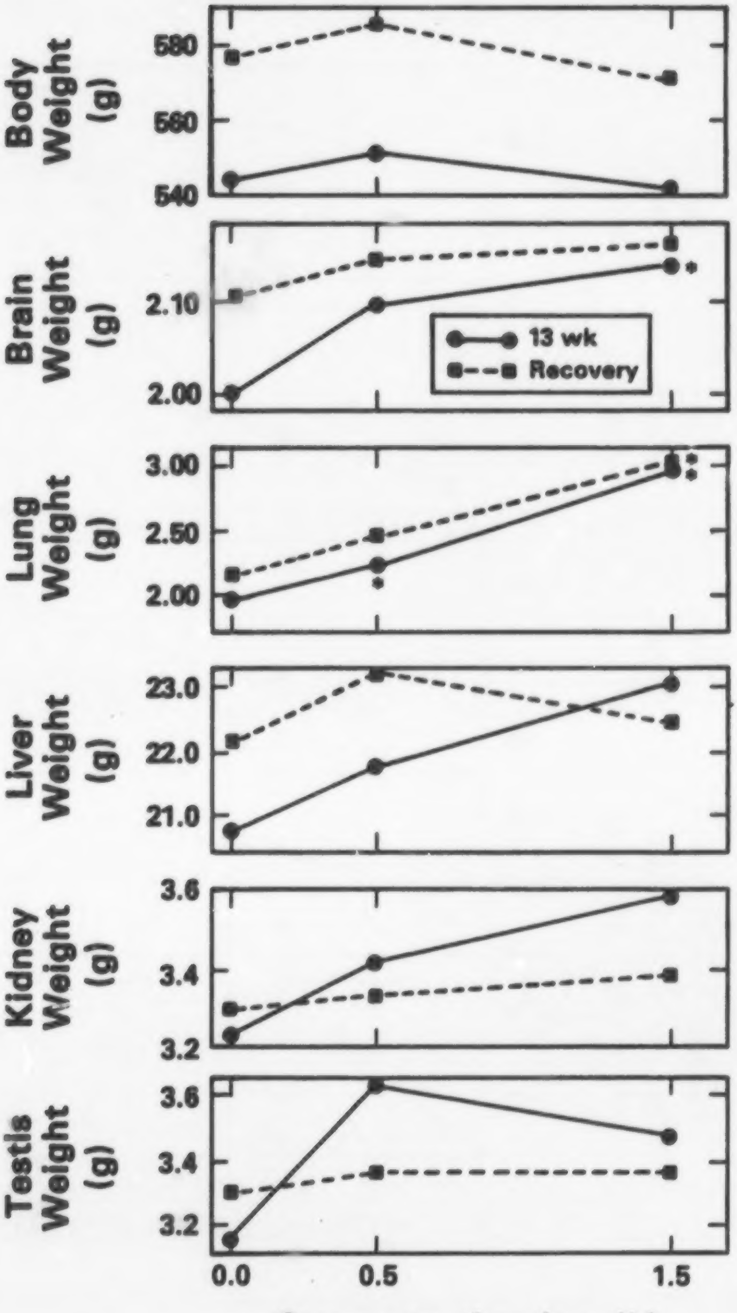

Figure 44. Effect of subchronic 13-wk exposure and 4-wk recovery period on body and tissue weights. *Significantly different from control ($p < 0.05$).

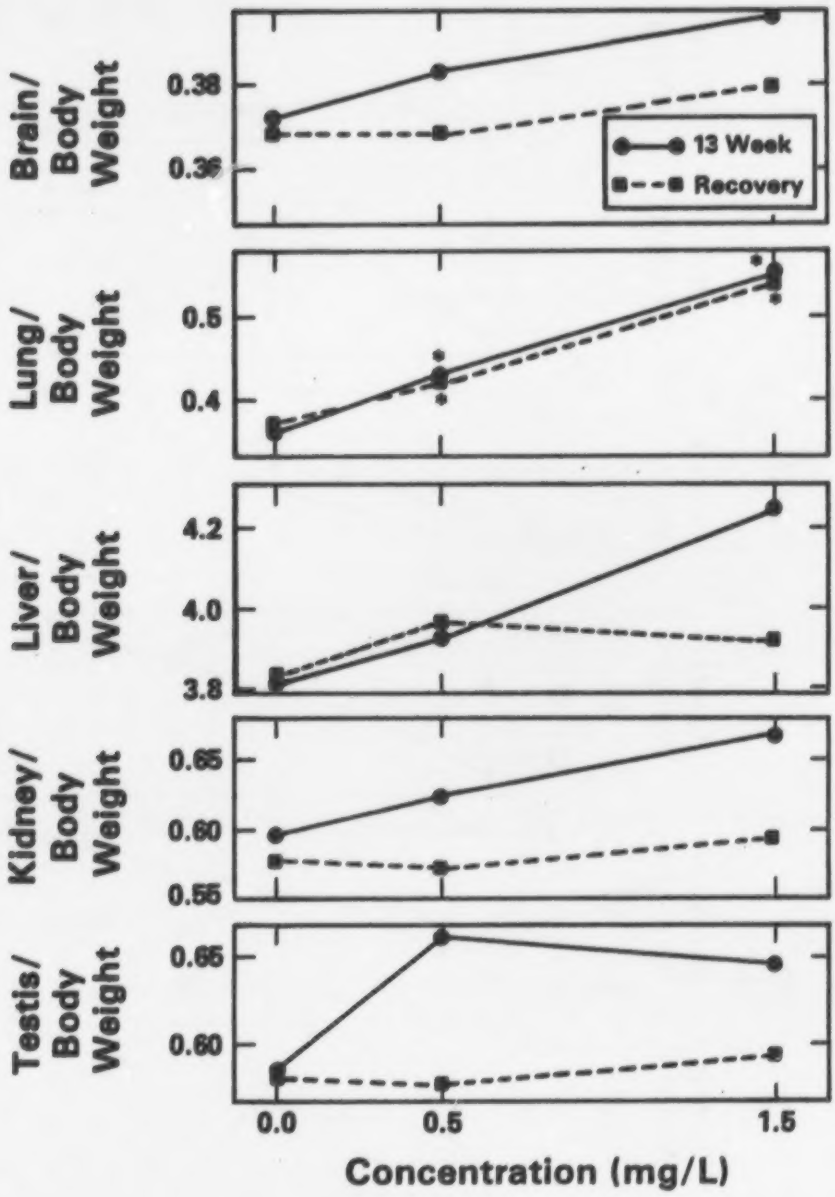

Figure 45. Effect of subchronic 13-wk exposure and 4-wk recovery period on tissue-to-body weight ratios. *Significantly different from control ($p < 0.05$).

Multivariate ANOVA of this same profile of ratios for recovery animals also indicated statistical significance ($p = 0.0001$, Wilks' criterion) due to concentration. Individual ANOVAs, however, indicated that only the lung-to-body weight ratio was affected by fog oil smoke concentration ($p = 0.0001$). Assuming a trend with increasing concentration, the Williams' test found only the mean at 1.5 mg/L different from that of controls for the same ratio. The other organ percentages did not even qualitatively follow a concentration-response relationship with fog oil smoke concentration.

A two-way multivariate ANOVA with fog oil smoke concentration and recovery time as factors was run on the combined data set. There was no significant interactive recovery time by fog oil smoke concentration effect on the joint response. The main effects of fog oil smoke concentration were significant but there was no multivariate effect due to recovery time. The univariate two-way ANOVAs indicated that the lung-to-body weight ratio was strongly affected by fog oil smoke concentration and that this effect still remained after recovery.

Pulmonary Physiology

Individual ANOVAs of each parameter indicated concentration-related changes in lung wet weight ($p = 0.0001$) and lung dry weight ($p = 0.0001$). Lung wet and dry weights were affected similarly after 13-wk exposure with and without the recovery period (Figure 46). Pooling over concentration groups, lung weights were lower for recovery animals. According to the Williams' test, there was still a significant upward trend in these weights, even for recovery animals, with the 1.5-mg/L concentration group significantly different from control. Williams' tests indicated that for lung dry weights, the means for both nonrecovery and recovery animals at 1.5 mg/L were significantly greater than their respective controls. For lung wet weight, a significant increase was also seen at the 1.5-mg/L level for both groups. No difference between any concentration group and control response could be seen for EEV using a two-sided Williams' test (Figure 46). However, using a less conservative subtest (a t test of the least squares means), there was a significant ($p = 0.028$) difference between the 1.5-mg/L and control groups of the nonrecovery animals.

Combining the recovery with the nonrecovery animals, the two-way multivariate ANOVA indicated both recovery ($p = 0.001$, Wilks' criterion) and concentration-related ($p = 0.0001$, Wilks' criterion) effects on the joint response. Individual two-way ANOVAs of each response showed differences, pooled over concentration groups, in N_2 washout levels due to recovery ($p = 0.0001$). Figure 47 shows that recovery animal levels of the N_2 washout parameter were lower than those for nonrecovery rats. The opposite effect was apparent for N_2 washout corrected for EEV; the slope for nonrecovery animals was generally lower ($p = 0.014$, Figure 47).

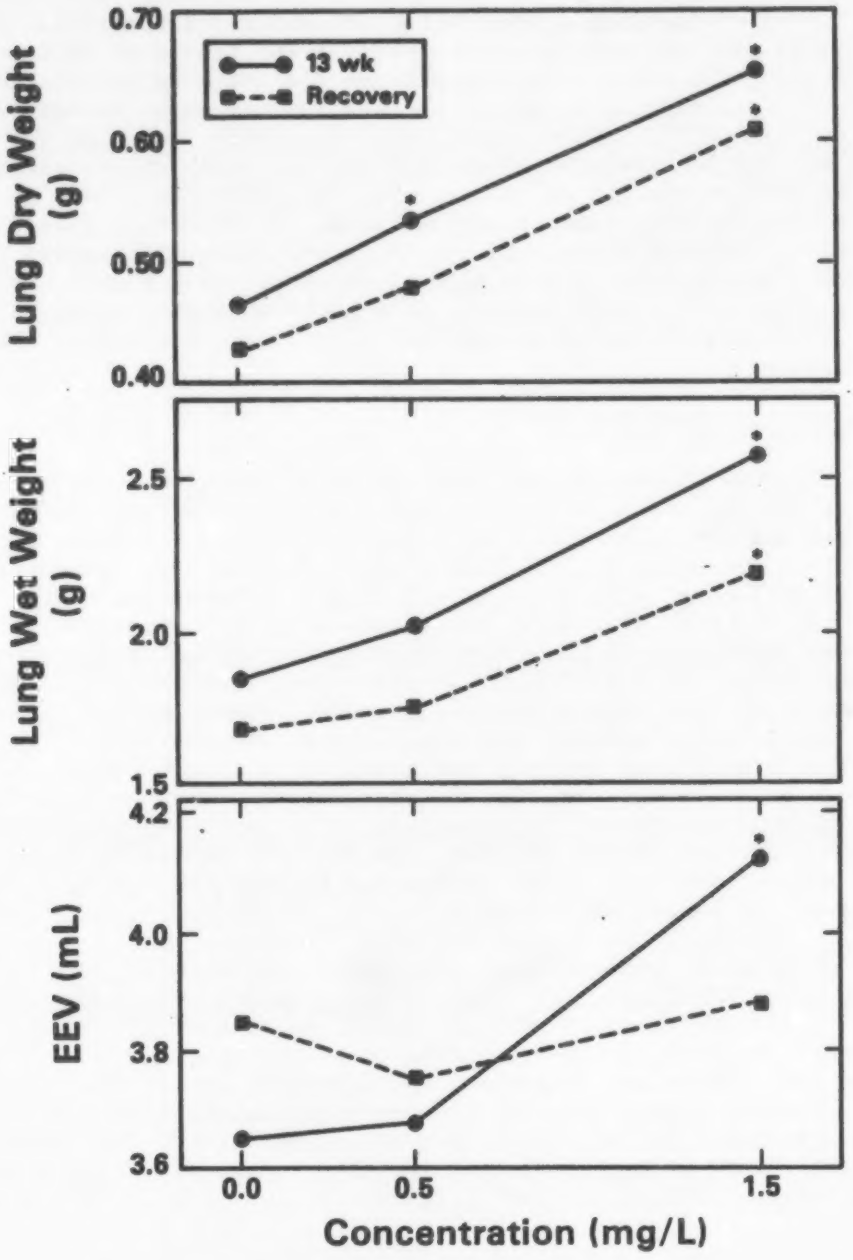

Figure 46. Effect of subchronic 13-wk exposure and a 4-wk recovery period on lung dry weight, lung wet weight, and EEV. *Significantly different from control ($p < 0.05$).

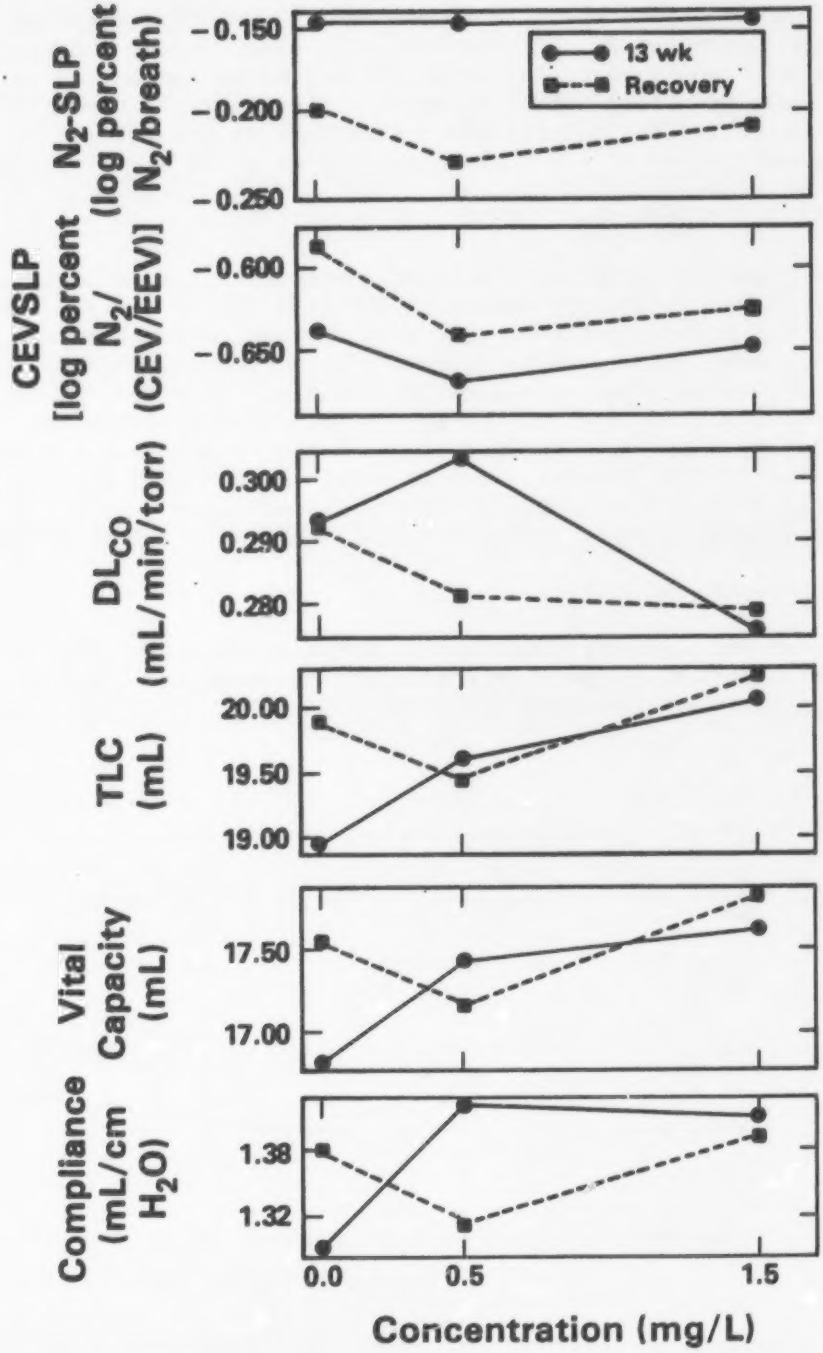

Figure 47. Effect of subchronic 13-wk exposure and a 4-wk recovery period on nitrogen washout slope (N_2-SLP), nitrogen washout slope corrected for EEV (CEVSLP), DL_{co}, TLC, vital capacity, and compliance.

Pulmonary Edema

Figure 48 shows the effects of fog oil smoke treatment on lavage fluid protein concentration. Recovery animals alone showed a marginal ($p = 0.082$) effect of fog oil smoke on lavage fluid protein concentration. Dunnett's test, however, could not distinguish either concentration group as different from controls. The two-way ANOVA indicated an interaction between recovery and concentration ($p = 0.038$) on the lavage fluid protein concentration.

Clinical Chemistry

For recovery animals, the joint response of the parameters studied did not appear to be affected by concentration ($p = 0.2168$, Wilks' criterion). Individual ANOVAs indicated that only amylase ($p = 0.025$) was influenced by fog oil smoke (Figure 49). Dunnett's test (two-sided) showed that the amylase level at 1.5 mg/L was significantly different from that of the controls. Triglyceride levels, which showed a significantly decreasing trend in nonrecovery animals, were indistinguishable in recovery animals (Figure 49). When data for the recovery animals were combined with that for the nonrecovery animals, there was an overall recovery effect ($p = 0.0001$, Wilks' criterion) but no concentration-related ($p = 0.3633$, Wilks' criterion) effect.

Xenobiotic Metabolism

There was a significant concentration-related decrease in zoxazolamine-induced paralysis time and an increase in AHH activity after

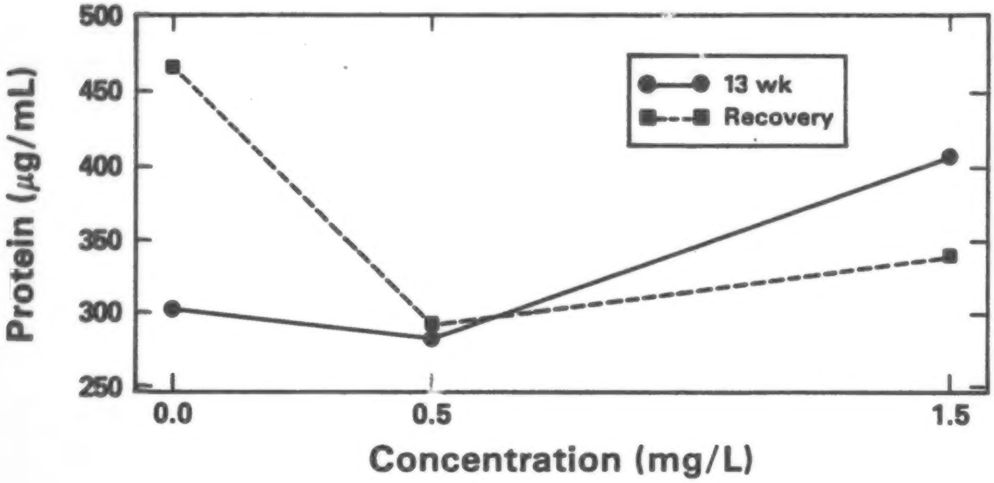

Figure 48. Effect of subchronic 13-wk exposure and a 4-wk recovery period on lavage fluid protein.

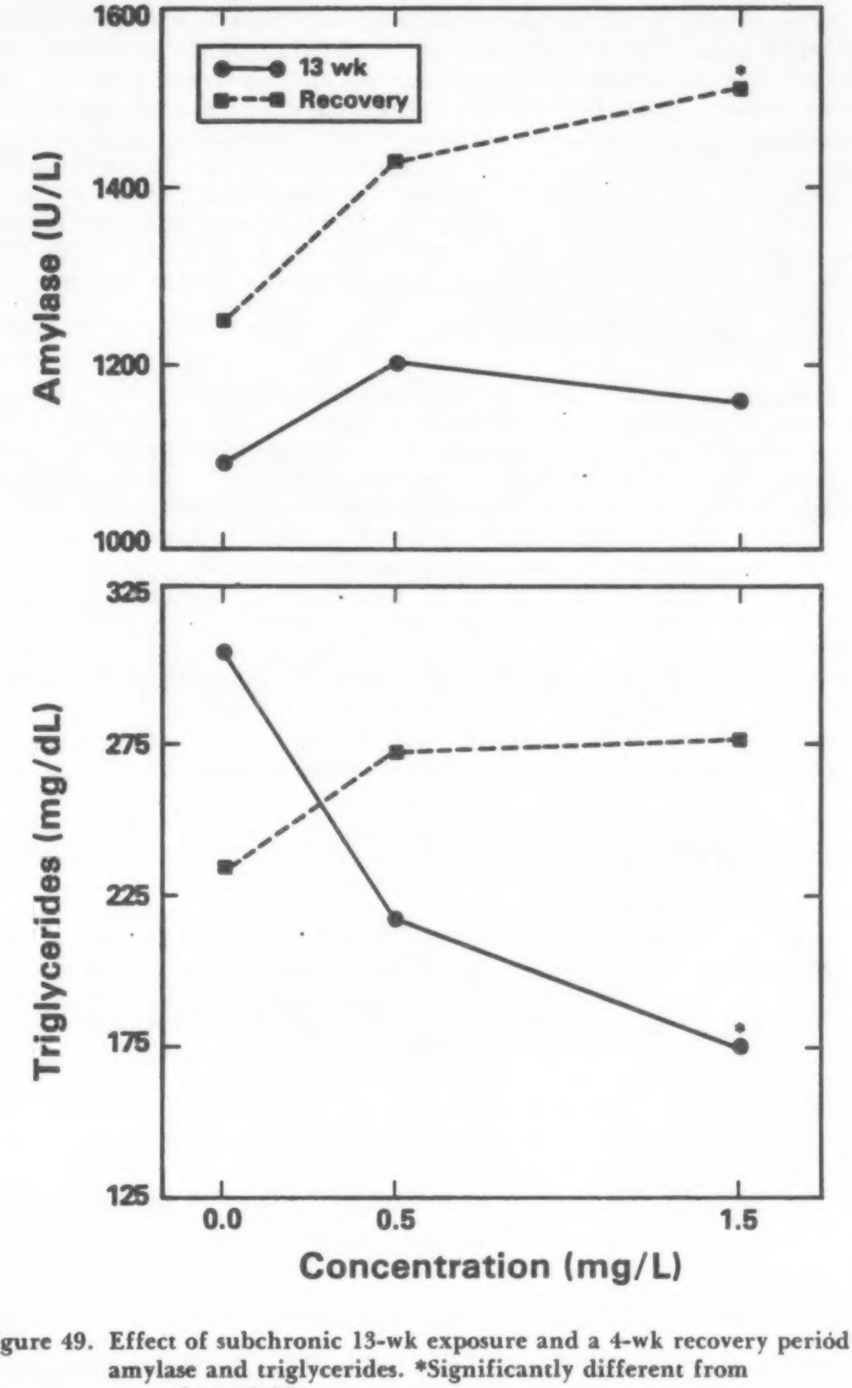

Figure 49. Effect of subchronic 13-wk exposure and a 4-wk recovery period on amylase and triglycerides. *Significantly different from control ($p < 0.05$).

13 wk of exposure. After the recovery period, AHH and paralysis time were measured to determine the duration of the effect observed immediately after the 13-wk exposure (Figure 50). AHH levels were log-transformed to correct for heterogeneity of variance as had been done for nonrecovery animals. Paralysis time and AHH activity were significantly affected after a 13-wk exposure but returned to normal during the recovery period. Among recovery period animals, paralysis time increased with concentration but not significantly as compared to nonrecovery animals that showed a significant decrease in paralysis time by concentration. The two-way ANOVA combining data for recovery and nonrecovery animals showed an obvious interaction ($p = 0.0001$) between the effects of recovery and concentration. Cytochrome P450 concentrations were not significantly altered by 13-wk exposure to fog oil smoke, nor did the recovery period show a latent effect (Figure 50).

Immunology

Immunology data (mitogen data and NK cell data) for recovery and nonrecovery animals were analyzed separately. Parameters of mitogen data were transformed by taking the square root of the difference between each mitogen and media count. Multivariate ANOVA of mitogen recovery data did not indicate any significant effect of fog oil smoke concentration on the joint response. The individual responses did not appear to be affected by fog oil smoke concentration either. No significant interaction (recovery time by fog oil smoke concentration) on the joint response was evident in the multivariate ANOVA of the combined data. Univariate ANOVA of individual responses showed significant effects of recovery on splenic Con A, PHA, and PWM responses (Figure 51). Recovery effects of borderline significance were present for peripheral blood PHA and Con A responses; no effect was shown for PWM (Figure 52). No main effect due to fog oil smoke concentration was indicated by either the multivariate or univariate analyses of the parameters.

A significant effect of fog oil smoke concentration on the joint response of the NK cell recovery data was indicated in the multivariate ANOVA ($p = 0.028$, Wilks' criterion). Effects of fog oil smoke concentration on individual responses were significant ($p < 0.02$) for NK25 and NK200 effector-to-target cell ratios (E/T) but not NK50 and NK100 (Figure 53). For the combined data, no significant interaction (fog oil smoke concentration by recovery) on the joint response of the parameters was evident. Significant main effects were indicated for both fog oil smoke concentration ($p = 0.0037$) and recovery ($p = 0.0001$). Consideration of individual responses by means of univariate ANOVA revealed significant main effects of both fog oil smoke concentration and recovery for NK E/Ts of 25, 100, and 200. For an NK E/T of 50, the effect of recovery on cytolysis was statistically significant.

Table 14 shows a summary of the results of Phase III - Subchronic Study: Part A.

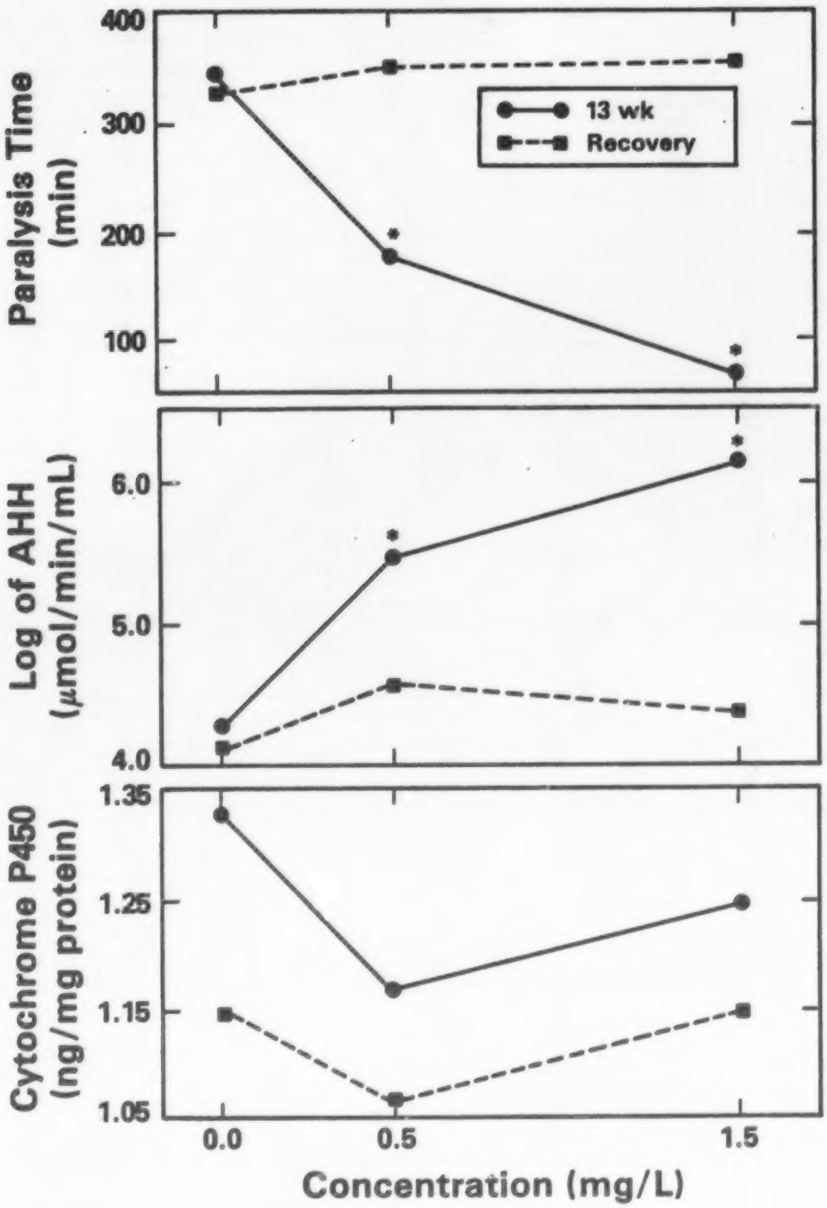

Figure 50. Effect of subchronic 13-wk exposure and a 4-wk recovery period on paralysis time, AHH activity, and cytochrome P450. *Significantly different from control ($p < 0.05$).

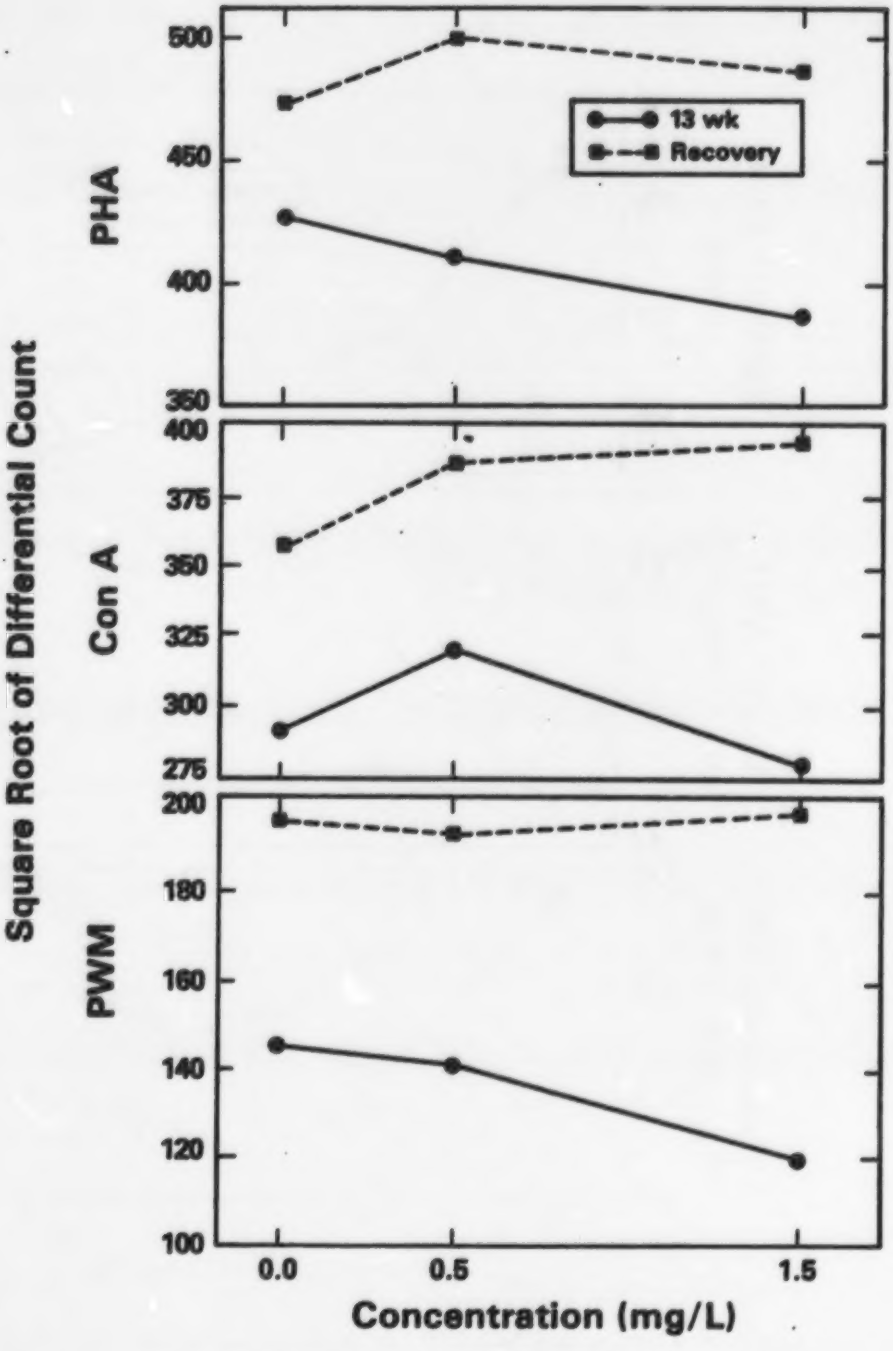

Figure 51. Splenic lymphocyte response to PHA, Con A, and PWM after a subchronic 13-wk exposure and a 4-wk recovery period.

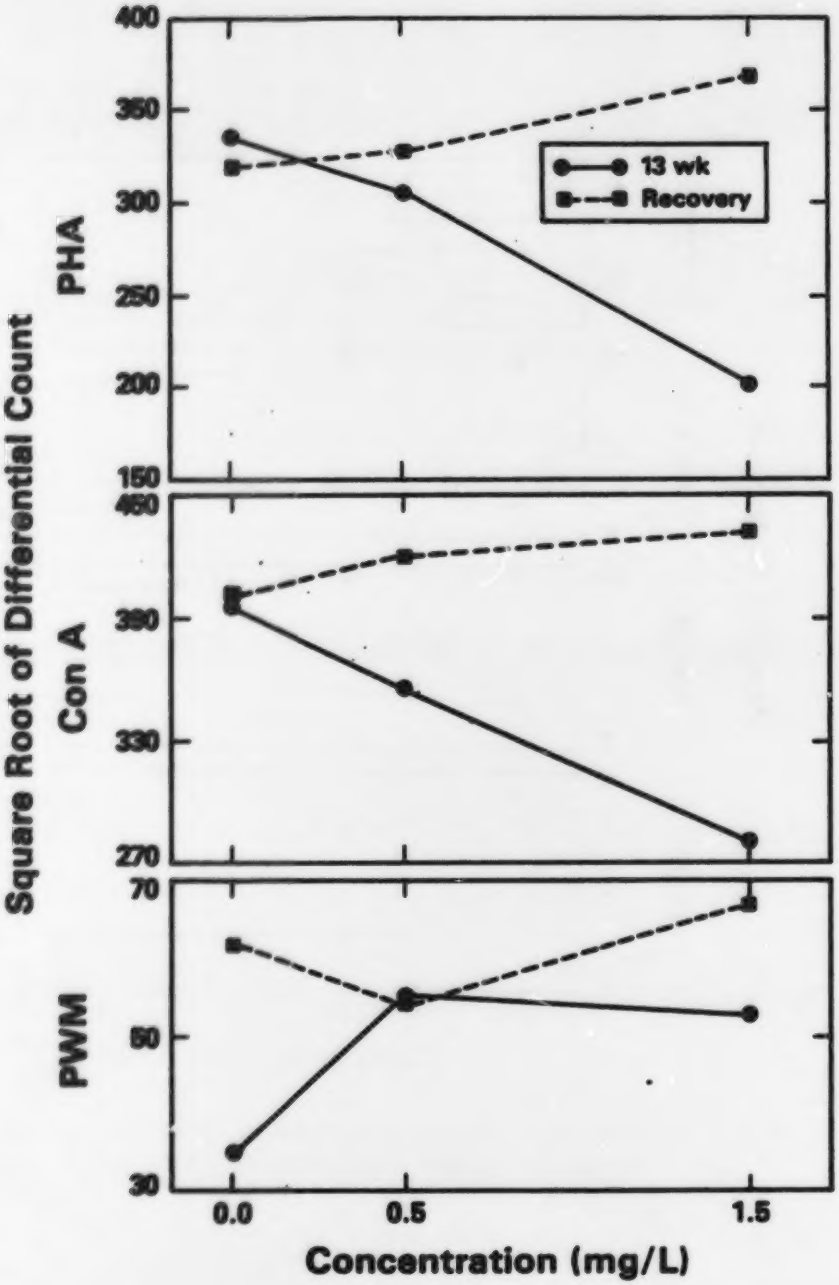

Figure 52. Peripheral blood cell response to PHA, Con A, and PWM after a subchronic 13-wk exposure and a 4-wk recovery period.

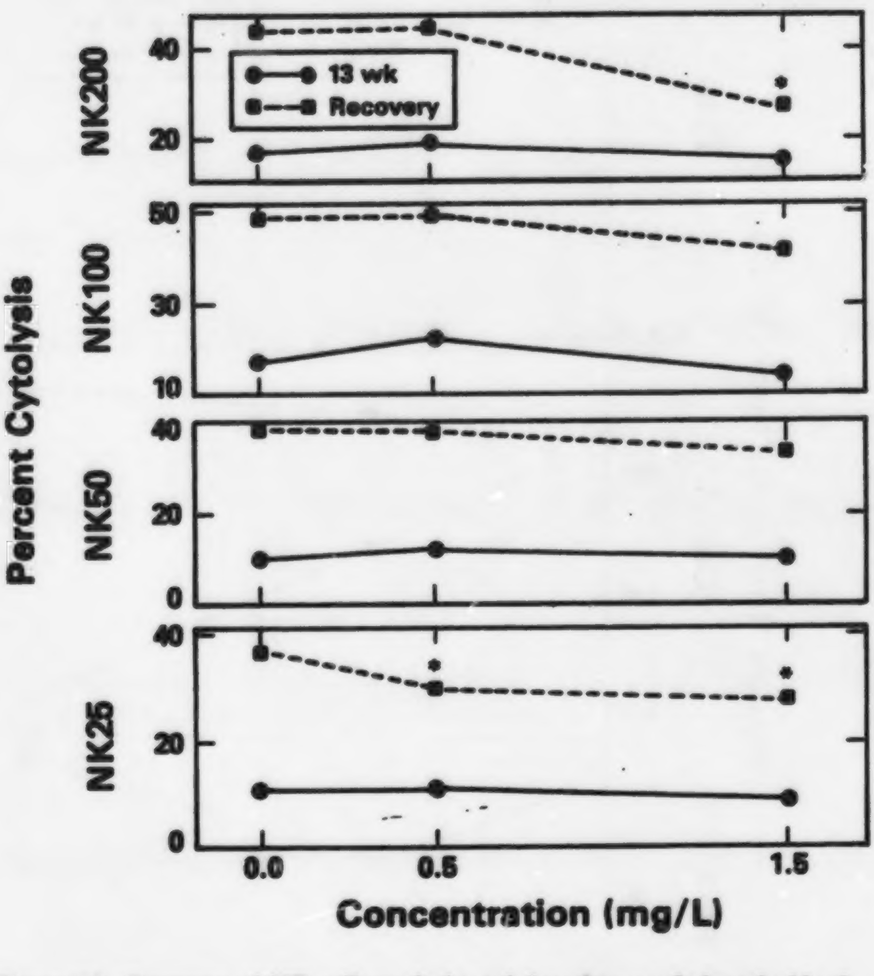

Figure 53. **Response of NK cell cytolytic activity after a subchronic 13-wk exposure and a 4-wk recovery period. *Significantly different from control ($p < 0.05$).**

TABLE 14. SUMMARY OF PHASE III - SUBCHRONIC STUDY: PART A

Parameter	Pulmonary Response 13-wk Exposure	4-wk Recovery Period
Histopathology	· Dose-related ↑ macrophages · Lymphoid hyperplasia	· Granulomatous pneumonia at 1.5 mg/L
Body weight	↓ at 1.5 mg/L	NS[a]
Lung weight	↑ at 0.5 and 1.5 mg/L	↑ at 1.5 mg/L
Pulmonary physiology	NS	NS
Pulomnary edema	NS	NS
	Systemic Response	
Histopathology	· Hyperplasia and congestion of mandibular lymph nodes at 0.5 and 1.5 mg/L	Similar to 13-wk exposure period data
Clinical chemistry	↓ Triglycerides	↑ Amylase
AHH activity	↑ at 0.5 and 1.5 mg/L	NS
Zoxazolamine-induced paralysis time	↓ at 0.5 and 1.5 mg/L	NS
Cytochrome P450	NS	NS
Immunology	NS	NS

a. NS = no significant effects.

Part B

Rats were exposed to either air, or 0.2 or 0.5 mg/L of fog oil smoke for 3.5 hr/day, 4 days/wk for 13 wk. This study was a follow-up to the Phase III Part A study, which had exposure levels of filtered air and 0.5 and 1.5 mg/L of fog oil smoke for 3.5 hr/day, 4 days/wk.

Histopathology

After terminal sacrifice, necropsies were conducted on three groups of 10 male rats that were exposed to 0.2 and 0.5 mg/L of fog oil smoke and to control air, respectively. Treatment-related microscopic changes were observed in the lungs of male rats exposed to 0.2 and 0.5 mg/L of fog oil smoke for 13 wk when compared to control groups. A diffuse accumulation of macrophages was present in the pulmonary alveoli. The degree of involvement was generally considered to be minimal to slight in rats exposed to 0.2 mg/L of fog oil smoke and to be slight to moderate in rats exposed to 0.5 mg/L of fog oil smoke. Other lesions observed in the respiratory tract that occurred only in rats exposed to fog oil smoke included submucosal and subacute inflammation in the larynx of 1 of 9 rats exposed to 0.5 mg/L, and submucosal lymphocytic infiltrates in the larynx of 2 of 10 rats exposed to 0.2 mg/L and 4 of 10 rats exposed to 0.5 mg/L of fog oil smoke. Multiple foci of macrophage accumulation in the cortical and medullary sinusoids observed in the peribronchial lymph node of one rat exposed to 0.5 mg/L was similar to that observed in earlier studies at higher concentrations.

At necropsy, the mandibular lymph nodes of several of the control and exposed rats were described as slightly enlarged and/or red. Microscopically, these lymph nodes had varying degrees of congestion of the cortical and medullary sinusoids. The incidence of congestion was similar in control and treated rats, and no relationships to exposure were present. Only mandibular lymph nodes described as abnormal at necropsy were examined histopathologically; therefore, this tissue was not examined for all rats in the study.

No treatment-related changes were present in other tissues examined in this study. A few incidental lesions such as chronic myocarditis, tubular regeneration in the kidney, peribronchial lymphocytic infiltrates in the lung, and focal chronic inflammation in the liver were occasionally observed. These changes were considered to be within normal limits for male rats of this age and strain.

Pulmonary Physiology

Two-way ANOVA on each response indicated either a sizable interaction between REP and CONC, or a main effect of REP for the following measures: N_2 washout slope (N_2-SLP, REP effect, $p = 0.0001$), TLC (REP effect, $p = 0.036$), lung dry weight (REP effect, $p = 0.008$). The data for the two replicates at 0.5 mg/L were combined for the following parameters: compliance, body weight, lung wet weights, DL_{co}, and vital capacity. When the two replicates were combined using 0.0 and 0.5 replicate data, body weight decreased with increasing concentration but not in a statistically notable fashion (Figure 54). However, at 1.5 mg/L, the observed decrease of 72 g is significant.

When the Williams' test was applied separately to each replicate's data, lung wet and dry weights showed significant trends with concentration (Figure 54). Other parameters were not significant (Figures 55 and 56).

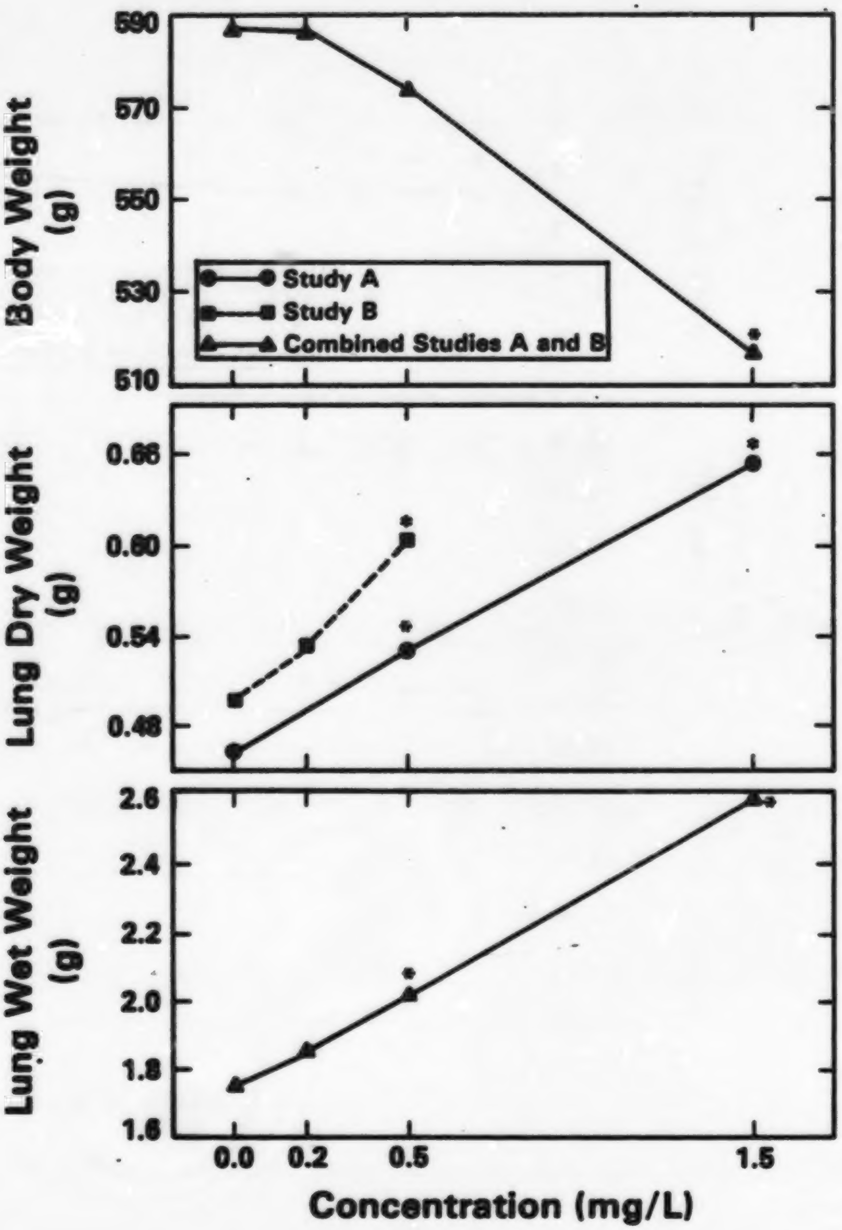

Figure 54. Concentration-response after subchronic 13-wk exposure on body weight, lung dry weight, and lung wet weight. *Significantly different from control ($p < 0.05$).

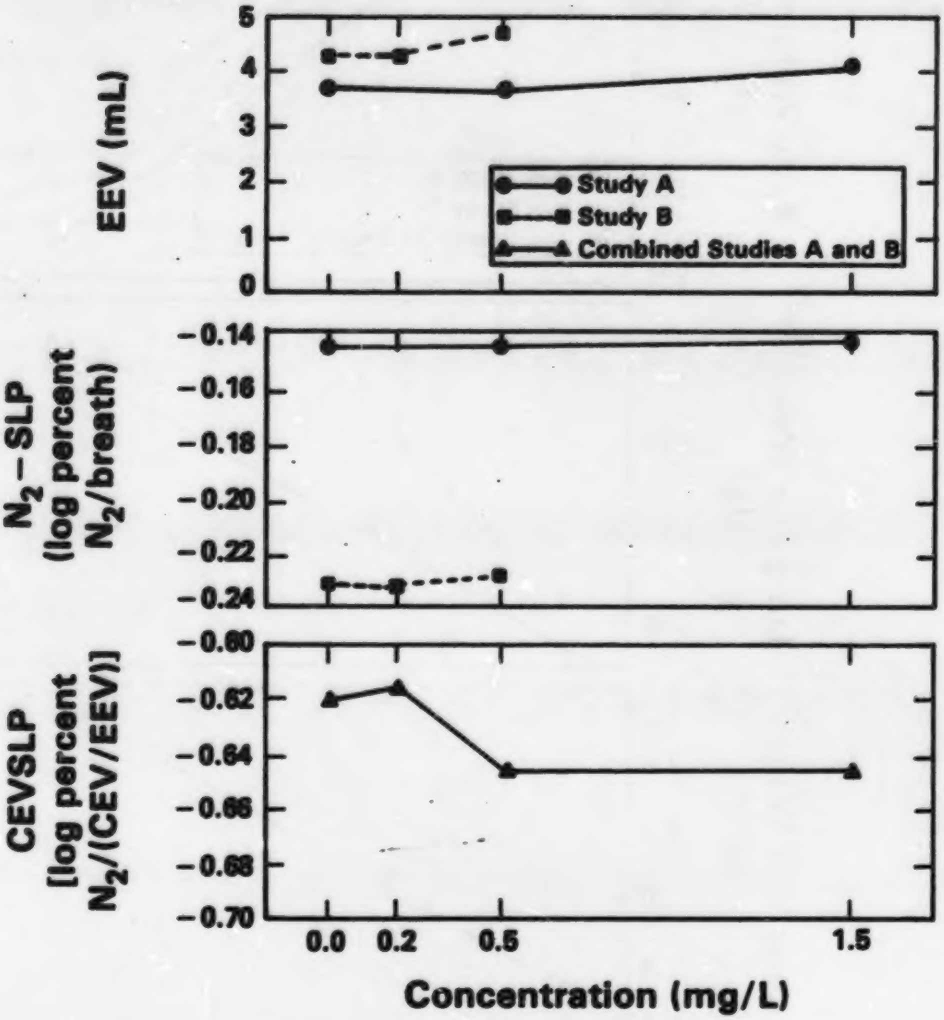

Figure 55. Concentration-response after subchronic 13-wk exposure on EEV, N_2-SLP, and CEVSLP.

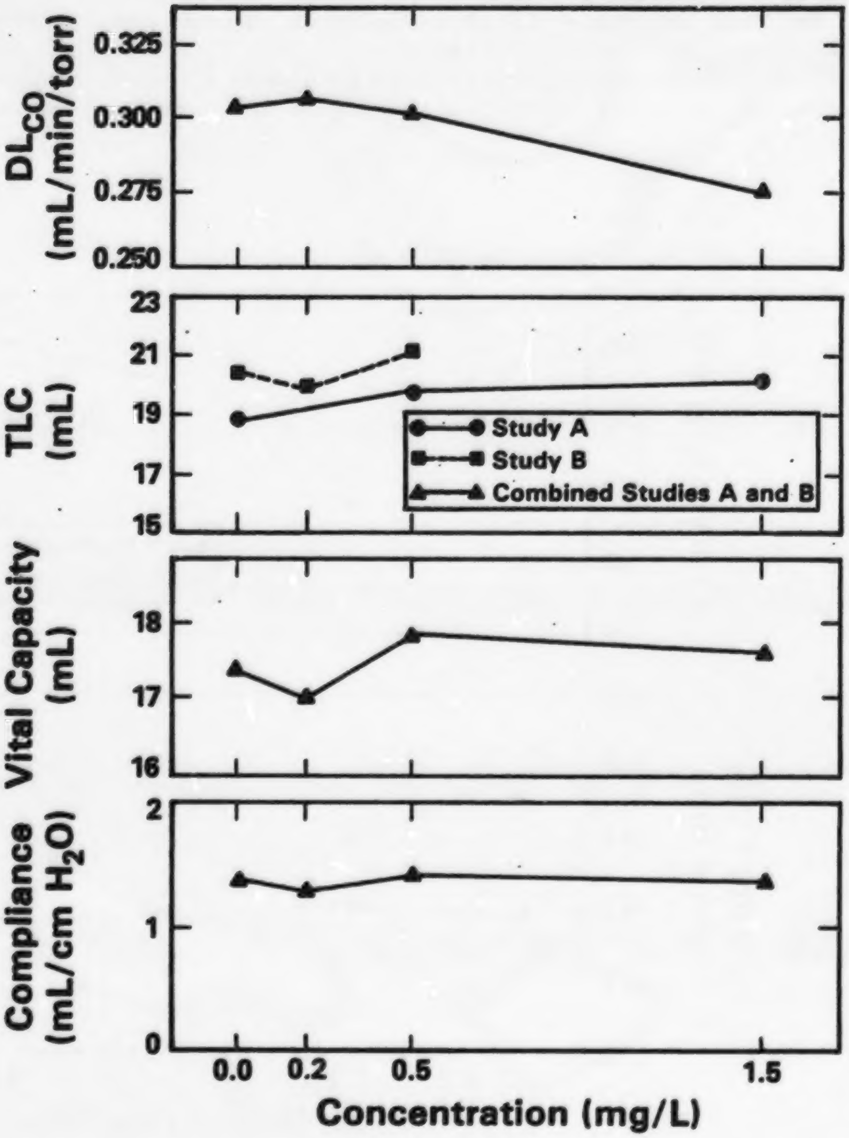

Figure 56. Concentration-response after subchronic 13-wk exposure on DL$_{CO}$, TLC, vital capacity, and compliance.

Pulmonary Edema

Three parameters were examined here: body weight, volume of lavage fluid removed, and lavage fluid protein concentration. The two-way ANOVAs of the data from both replicates for filtered air and at 0.5 mg/L indicated sizable replicate differences for both lavage fluid volume and lavage fluid protein. Thus, the data for these two variables from the two replicates were not combined prior to running Williams' test. Williams' test on lavage fluid protein for the second replicate indicated that the mean response at 0.5 mg/L was different from the control mean (Figure 57). Because there was evidence

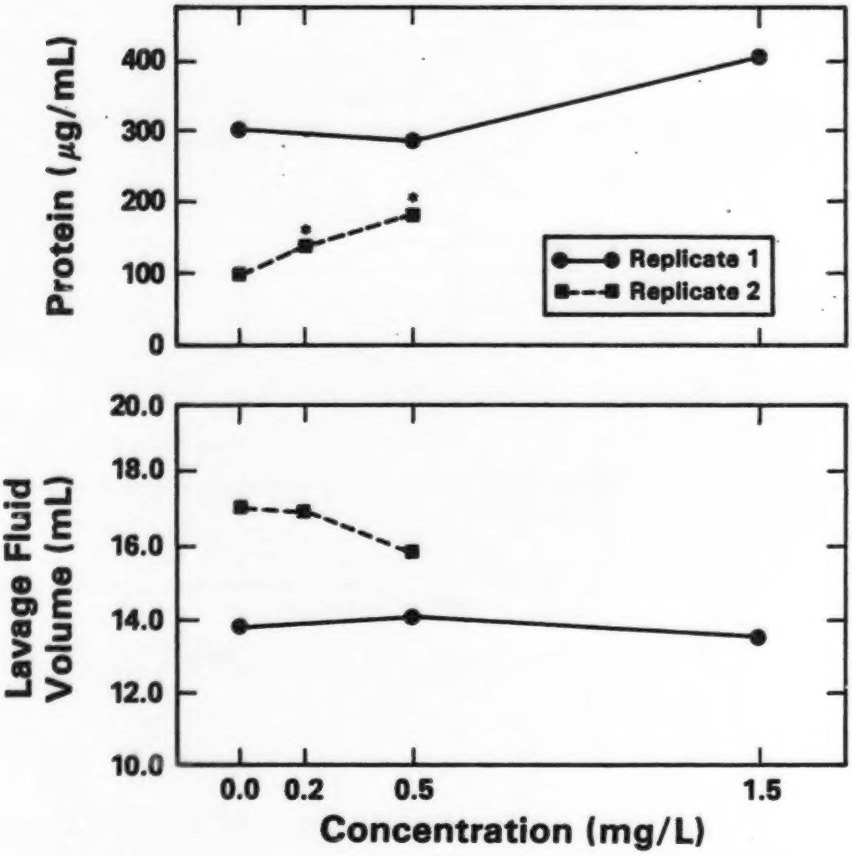

Figure 57. Concentration-response after subchronic 13-wk exposure on lavage fluid protein and volume of lavage fluid recovered. *Significantly different from control ($p < 0.05$).

of nonhomogeneity of concentration group variances, the same analysis was repeated by the ranked observations. This showed that the mean rank of the 0.2-mg/L group was also different from that of control. This indicated that the nominal significance probability of 5% was affected by the homogeneity problem. There was no difference in the volume of lavage fluid recovered at each concentration (Figure 57).

Pulmonary Cells

Five parameters were analyzed: total cells, the percentage of total cell viability, and percentages of macrophages, PMNs, and lymphocytes. These end points were not examined in Phase III Part A; therefore, no combining of replicates was involved in this analysis. The percentage of total cell viability was not affected by fog oil smoke concentration at any level (Figure 58). Percentages of macrophages, PMNs, and lymphocytes, however, were affected, with 1.5 mg/L being the lowest effective concentration. There was some heterogeneity among concentration group variances for macrophages and PMNs, but analysis of the ranks of these variables yielded the same results.

Hematology

This end point was not examined in Phase III Part A. Information was collected on 11 blood parameters, 9 of which had sufficient information to analyze. A one-way ANOVA by fog oil smoke-concentration was run against each of these nine responses. None could be detected as significantly affected by concentration. Hemoglobin showed a general increase with concentration but was not significantly affected (data not shown).

Xenobiotic Metabolism

Zoxazolamine-Induced Paralysis Time

The parameters examined for this end point were induction time and paralysis time. The two-way ANOVA on paralysis time indicated an ($p = 0.017$) interaction between REP and CONC. Thus, the data for paralysis time were not pooled over both replicates. Even when examined separately by replicate (Figure 59), however, the mean paralysis time of 189.5 min at 0.2 mg/L is significantly different from the control mean of 270.6 min with Williams' test. There was no effect of increasing concentration on induction time (data not shown).

Enzyme Activity

From the two-way ANOVAs with REP and CONC as factors, there was an interaction involving REP for cytochrome P450. When we looked at the cytochrome P450 concentration response separately by REP with Williams' test, there was no indication of a statistically significant concentration-related trend (data not shown). AHH showed a significant increasing trend with concentration, and the mean response at even 0.2 mg/L was significantly different from control (Figure 59). However, there were some heterogeneous

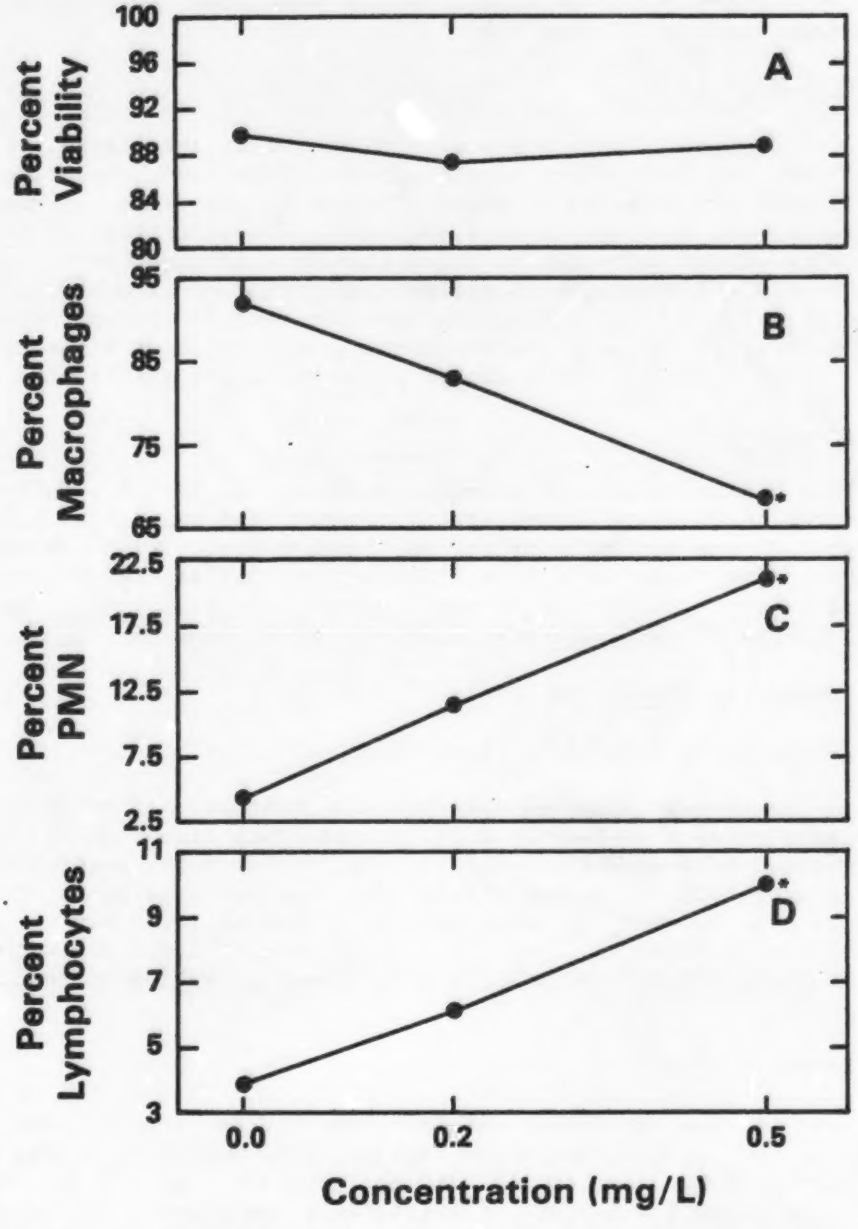

Figure 58. Concentration-response after subchronic 13-wk exposure on percent viability, percent macrophages, percent PMNs, and percent lymphocytes. *Significantly different from control ($p < 0.05$).

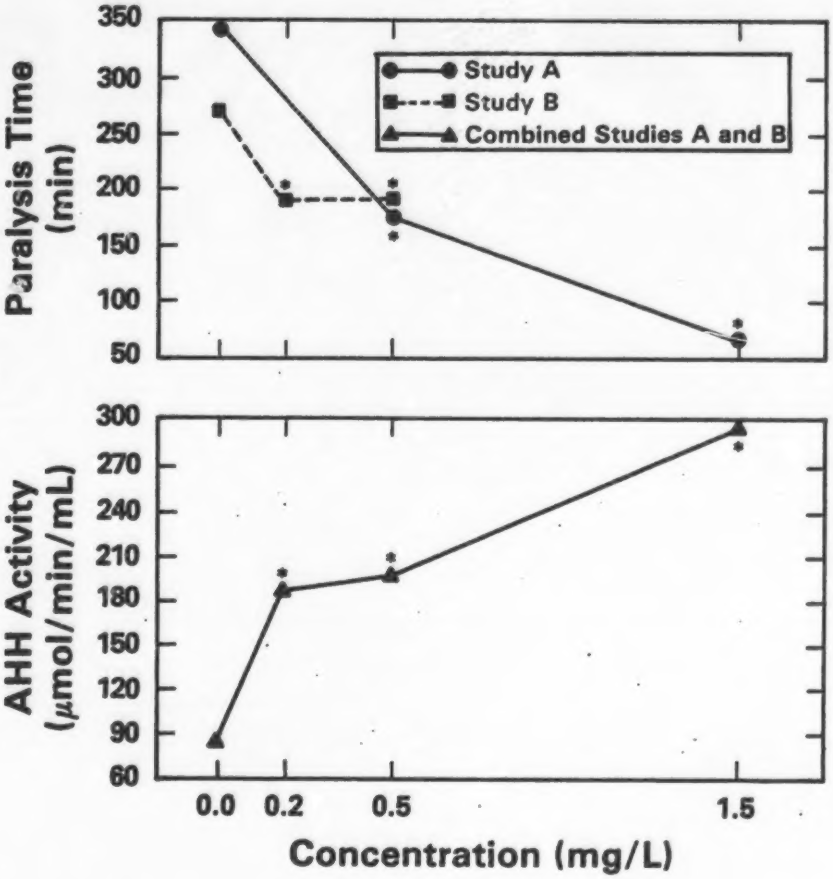

Figure 59. Concentration-response after subchronic 13-wk exposure on paralysis time and AHH activity. *Significantly different from control ($p < 0.05$).

variances among the different concentration groups. The analysis of AHH was repeated on the ranks of the AHH levels to check the results under more reasonable ANOVA assumptions. When Williams' test was applied to the ranks, the maximum likelihood estimates of the mean ranks at 0.2, 0.5, and 1.5 mg/L were indistinguishable, but all were significantly higher than control.

Table 15 shows a summary of the results from Phase III - Subchronic Study: Part B.

Part C

Male and female rats were exposed to filtered air or 1.5 mg/L of fog oil smoke for 13 wk to compare the sex response at the 1.5-mg/L concentration.

TABLE 15. SUMMARY OF PHASE III - SUBCHRONIC STUDY: PART B

Parameter	0.2 mg/L	0.5 mg/L
Pulmonary Response		
Histopathology	Mild ↑ macrophages	Moderate ↑ macrophages
Body weight (c)[a]	NS[b]	NS
Dry lung weight (s)[c]	NS	↑
Pulmonary physiology (c)	NS	NS
Pulmonary edema	↑[d]	↑[d]
Pulmonary cells	NS	↑ % PMNs ↓ % macrophages ↑ % lymphocytes
Systemic Response		
Histopathology	NS	NS
Hematology	NS	NS
AHH activity	↑	↑
Zoxazolamine-induced paralysis time	↓	↓
Cytochrome P450	NS	NS

a. (c) = combined replicate data analysis

b. NS = no significant effects.

c. (s) = single replicate data analysis.

d. Only occured in replicate 2.

Histopathology

Treatment-related microscopic changes were observed in the lungs and peribronchial lymph nodes of rats of both sexes exposed to 1.5 mg/L of fog oil smoke for 13 wk when compared to control groups. A diffuse accumulation of macrophages were present in the pulmonary alveoli. The degree of involvement was slightly greater in the male rats than female rats exposed to 1.5 mg/L of fog oil smoke. Most of the male rats had a moderate degree of diffuse alveolar macrophages; female rats were generally considered to have only slight involvement. Although the distribution of macrophages involved all regions of the pulmonary parenchyma, there was an increased number of alveolar macrophages near the terminal bronchioles. Other changes observed in the lungs that appeared to be associated with exposure to fog oil smoke included multifocal granulomatous pneumonia in 3 of 10 males, focal adenomatous hyperplasia in 1 of 10 female rats, and congestion and perivascular edema in 3 female rats that died during the study. Multiple foci of macrophage accumulation were present in the cortical and medullary sinusoids of the peribronchial lymph nodes of 5 of 10 male and 5 of 10 female rats exposed to fog oil smoke.

At necropsy, the mandibular lymph nodes of several of the control and exposed rats were described as slightly enlarged and red. Microscopically, these lymph nodes had varying degrees of congestion of the cortical and medullary sinusoids. The incidence of congestion was similar in control and treated rats of both sexes and no relationships to exposure were present. Only mandibular lymph nodes described as abnormal at necropsy were examined histopathologically; therefore, this tissue was not examined from all rats in the study.

No treatment-related changes were present in other tissues examined in this study. A few incidental lesions such as chronic myocarditis, tubular regeneration in the kidney, peribronchial lymphocytic infiltrates in the lung, and seminiferous tubular atrophy in the testes were occasionally observed. These changes were considered to be within normal limits for male rats of this age and strain.

Pulmonary Physiology

The same parameters that had been examined in other phases of the study were also examined here. Multivariate ANOVA indicated overall effects of gender ($p < 0.001$) and of concentration ($p < 0.001$) on the vector comprised of these 10 responses. No multivariate interaction of gender and concentration was evident for this overall response. When tested on an individual response basis, all the parameters indicated a main effect due to gender ($p < 0.001$), males' being greater than females' (Figures 60-62). Residual volume indicated a gender by concentration interaction ($p = 0.044$). When subtesting for concentration effect by gender using standardized differences of least square means, we observed a significant increase in residual volume in males ($p = 0.003$) but not in females ($p = 0.619$). Overall, both sexes appear equally sensitive to fog oil smoke exposure.

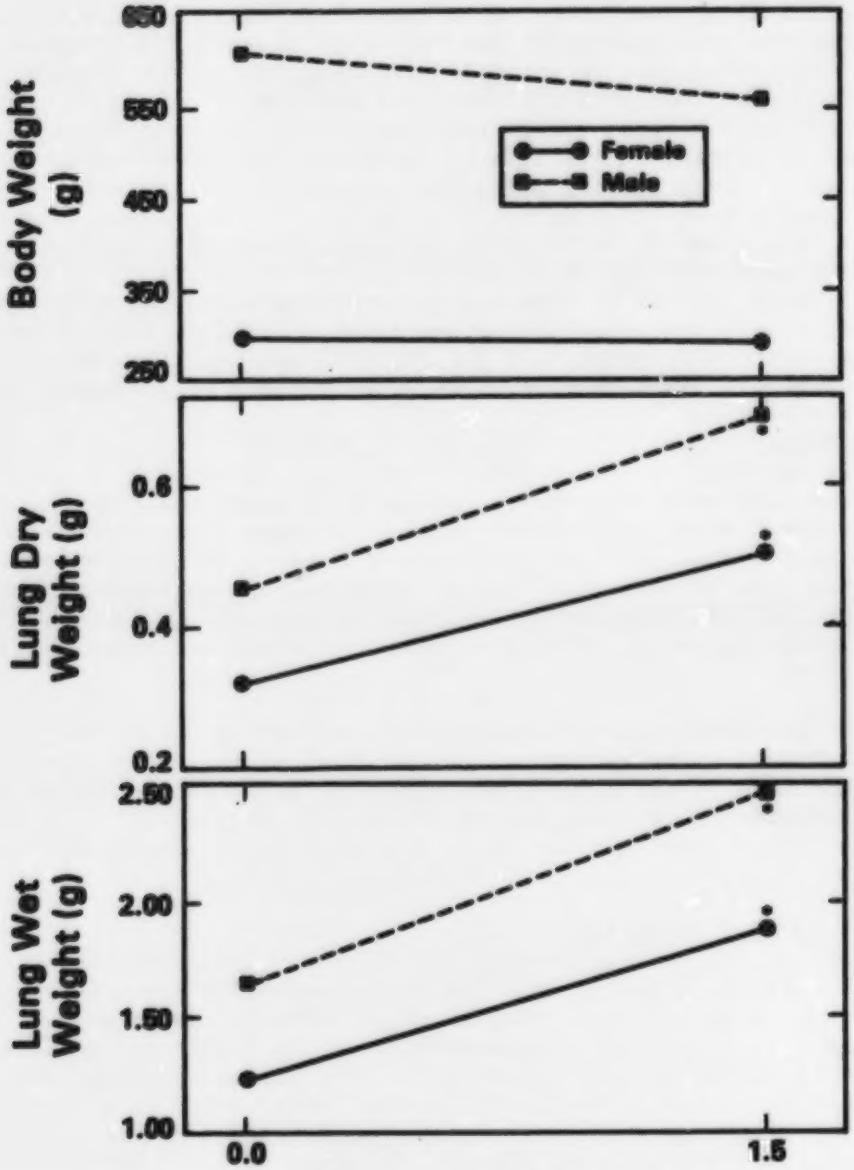

Figure 60. Comparison of female-male response to subchronic exposure on body weight, lung dry weight, and lung wet weight. *Significantly different from control ($p < 0.05$).

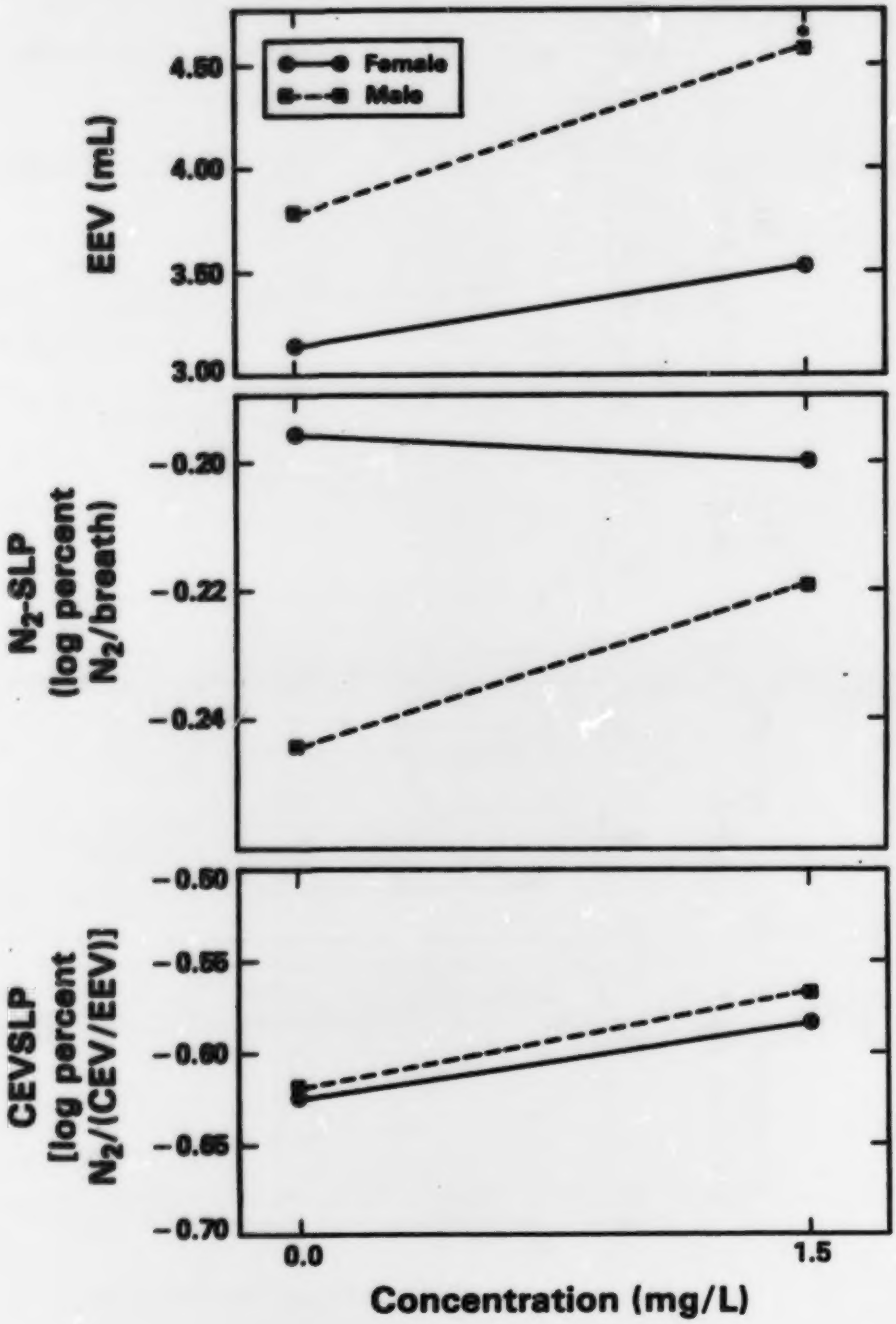

Figure 61. Comparison of female-male response to subchronic exposure on EEV, N_2-SLP, and CEVSLP. *Significantly different from control ($p < 0.05$).

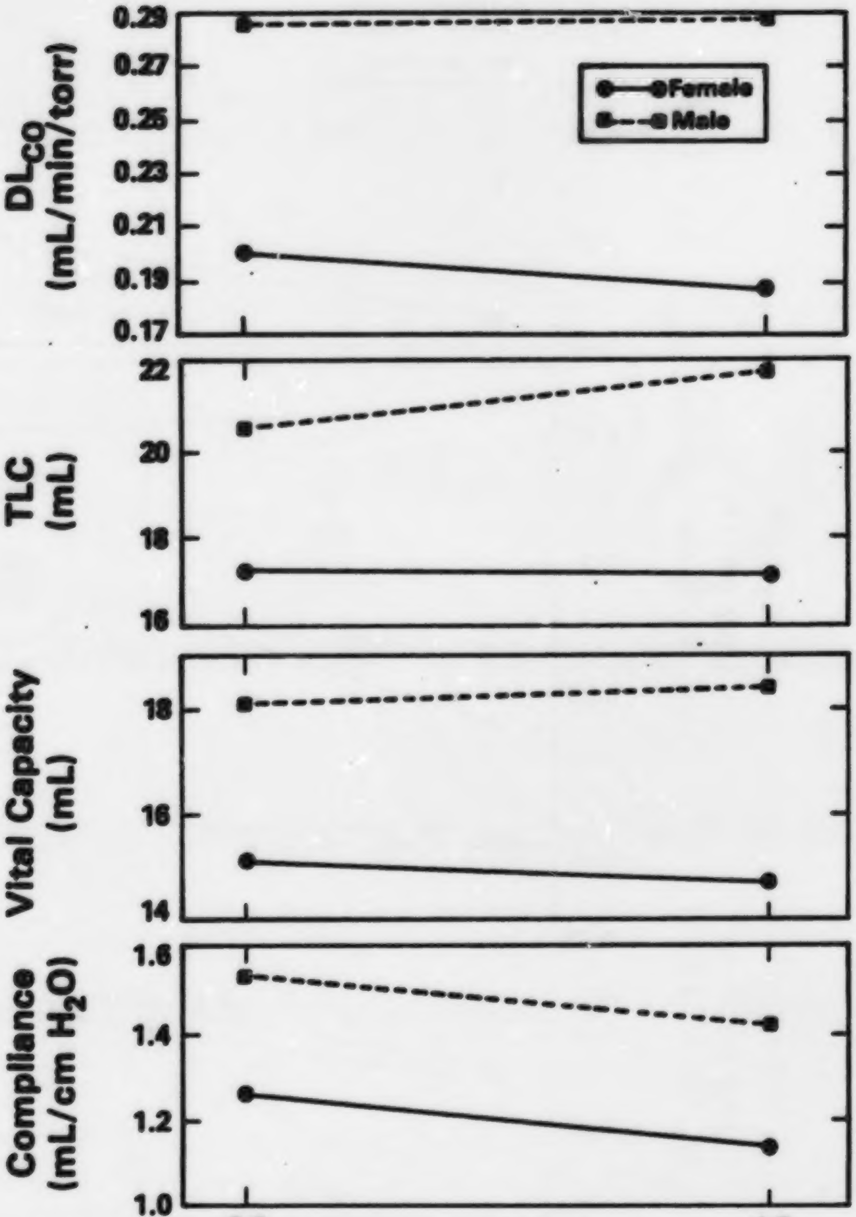

Figure 62. Comparison of female-male response to subchronic exposure on DL$_{CO}$, TLC, vital capacity, and compliance.

Pulmonary Edema

ANOVA of protein levels showed no gender effect ($p = 0.627$) but a strong fog oil smoke effect ($p < 0.001$, Figure 63). Protein levels increased more in females ($p < 0.001$) than in males ($p < 0.039$). Lavage fluid volume was affected only by gender and not by fog oil smoke exposure.

Pulmonary Cells

Each of the parameters was analyzed in a two-way ANOVA. No interactions involving gender and fog oil smoke were detectable. Total cells showed a gender effect but no effect due to fog oil smoke concentration (Figure 64). A strong ($p < 0.001$) effect of fog oil smoke, but no gender effect, was seen in both percentages of macrophages and of PMNs. The percentage of lymphocytes was affected by both gender ($p = 0.053$) and fog oil smoke ($p = 0.042$). No effects of either gender or fog oil smoke were detectable with respect to percentages of viability.

Hematology

From the two-way ANOVAs, there was evidence of gender and fog oil smoke concentration interactions for MCV, MCHC, and the percentage of hematocrit. For MCV, when subtesting by gender, levels dropped significantly ($p = 0.022$) in females but not in males whose levels increased although not significantly ($p = 0.212$, Figure 65). For the percentage of hematocrit, we saw a significant ($p = 0.006$) decrease in females but no change in males. With no interaction involving gender, the following showed an effect of concentration when pooled over sex: RBC ($p = 0.020$) and hemoglobin ($p = 0.031$, Figure 66). RBC also showed a significant ($p < 0.001$) gender effect. WBC and MCH were affected only by gender.

Xenobiotic Metabolism

Zoxazolamine-Induced Paralysis Time

Univariate ANOVA of paralysis time indicated a strong fog oil smoke effect ($p = 0.001$) but no effect of gender ($p = 0.815$, Figure 67). Subtests of the concentration effect showed that paralysis time decreased more in females ($p < 0.001$) than in males ($p = 0.053$) for this study.

Enzyme Activity

There was no evidence of a gender by fog oil smoke concentration interaction for any of the variables tested. AHH activity was affected by exposure to fog oil smoke. This was true when pooling over gender ($p < 0.001$) or when examining males ($p < 0.001$) and females ($p < 0.001$) separately (Figure 67). No changes due to gender or to fog oil smoke were detectable in cytochrome P450 levels.

Table 16 summarizes the results of Phase - III Subchronic Study: Part C.

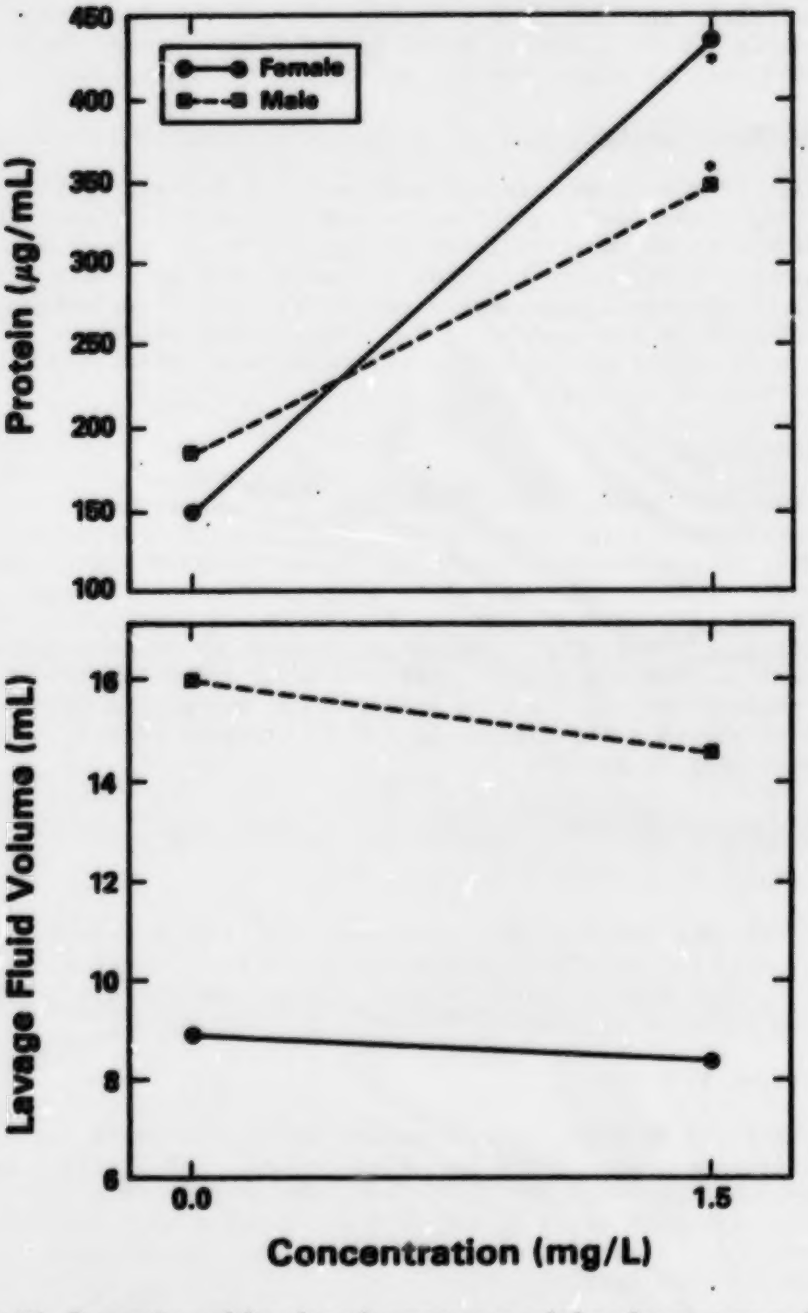

Figure 63. Comparison of female-male response to subchronic exposure on lavage fluid protein and volume. *Significantly different from control ($p < 0.05$).

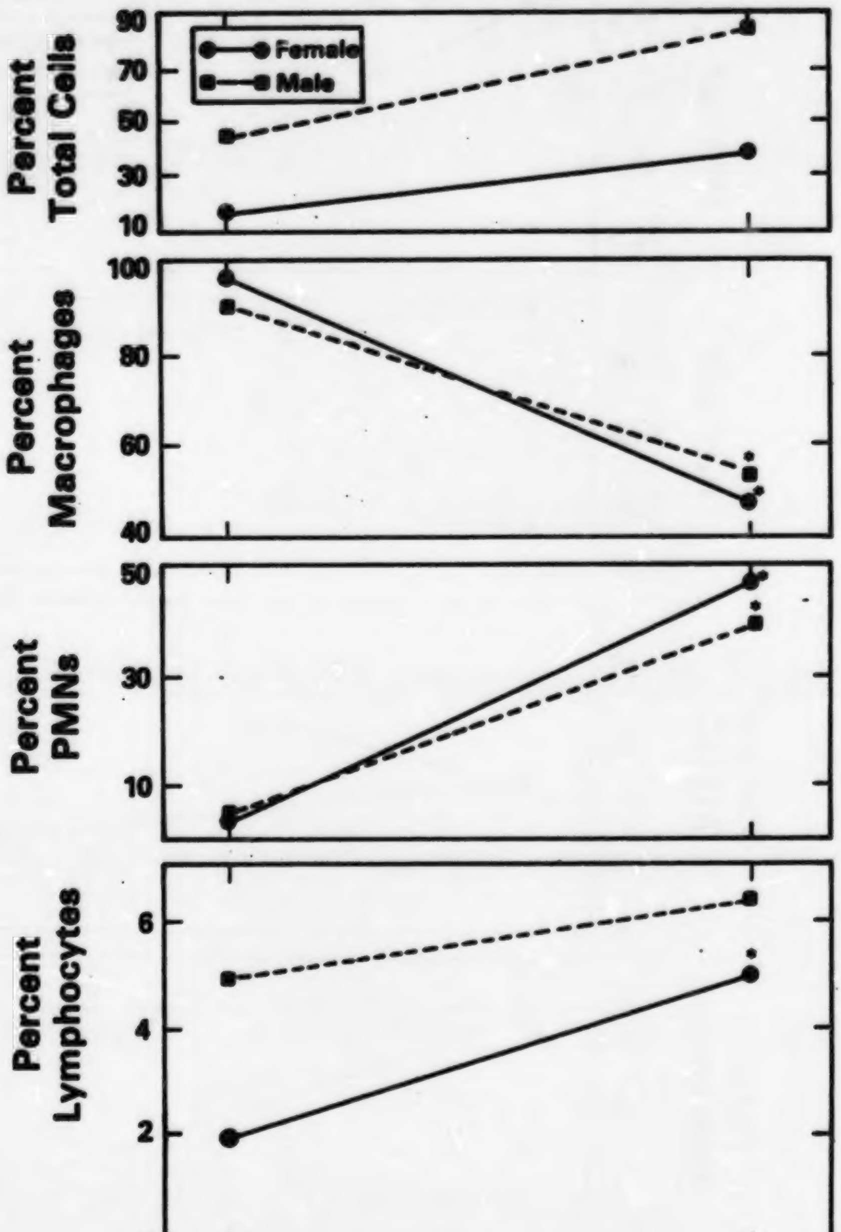

Figure 64. Comparison of female-male response to subchronic exposure in percent total cells, percent macrophages, percent PMNs, and percent lymphocytes. *Significantly different from control ($p < 0.05$).

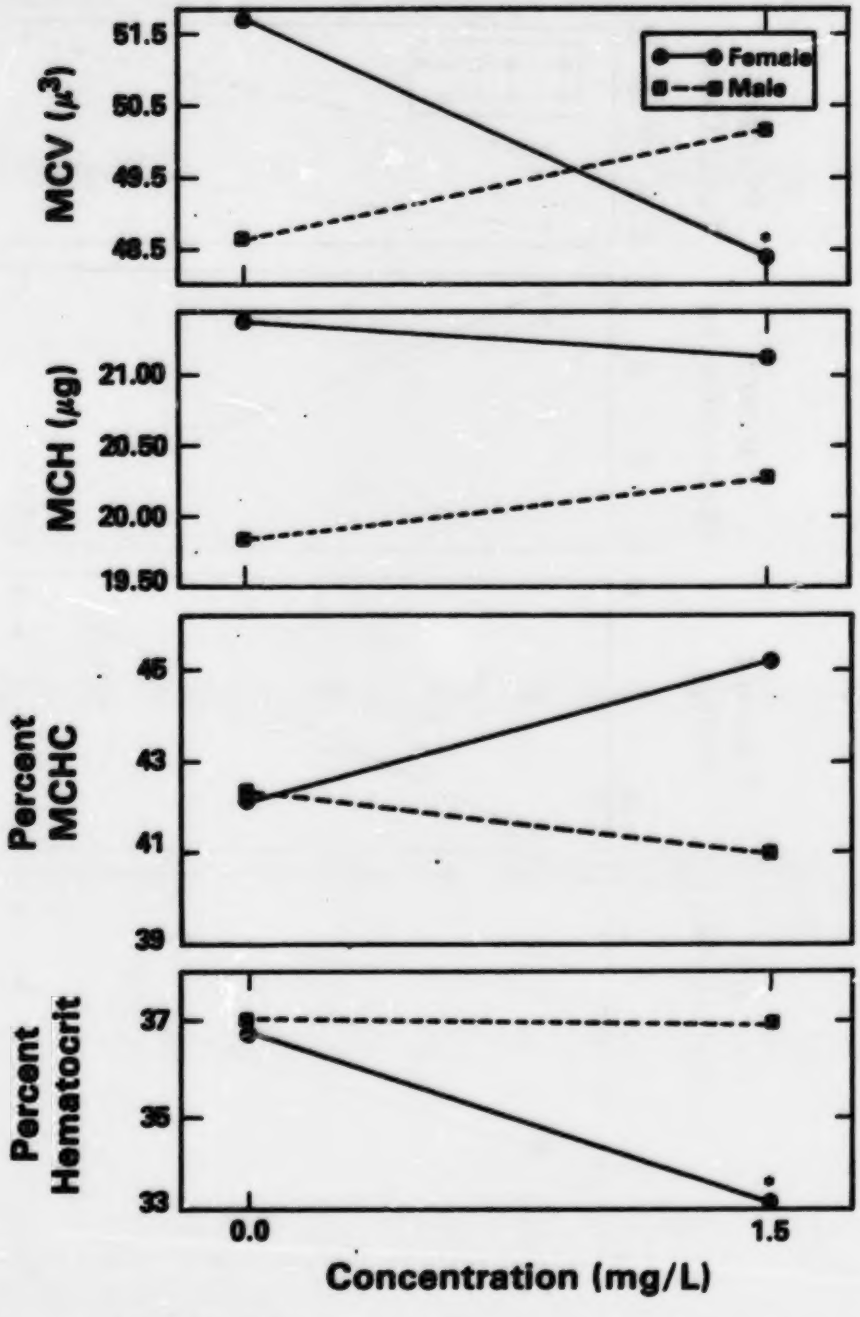

Figure 65. Comparison of female-male response to subchronic exposure on MCV, MCH, percent mean corpuscular hemoglobin concentration (MCHC), and percent hematocrit. *Significantly different from control ($p < 0.05$).

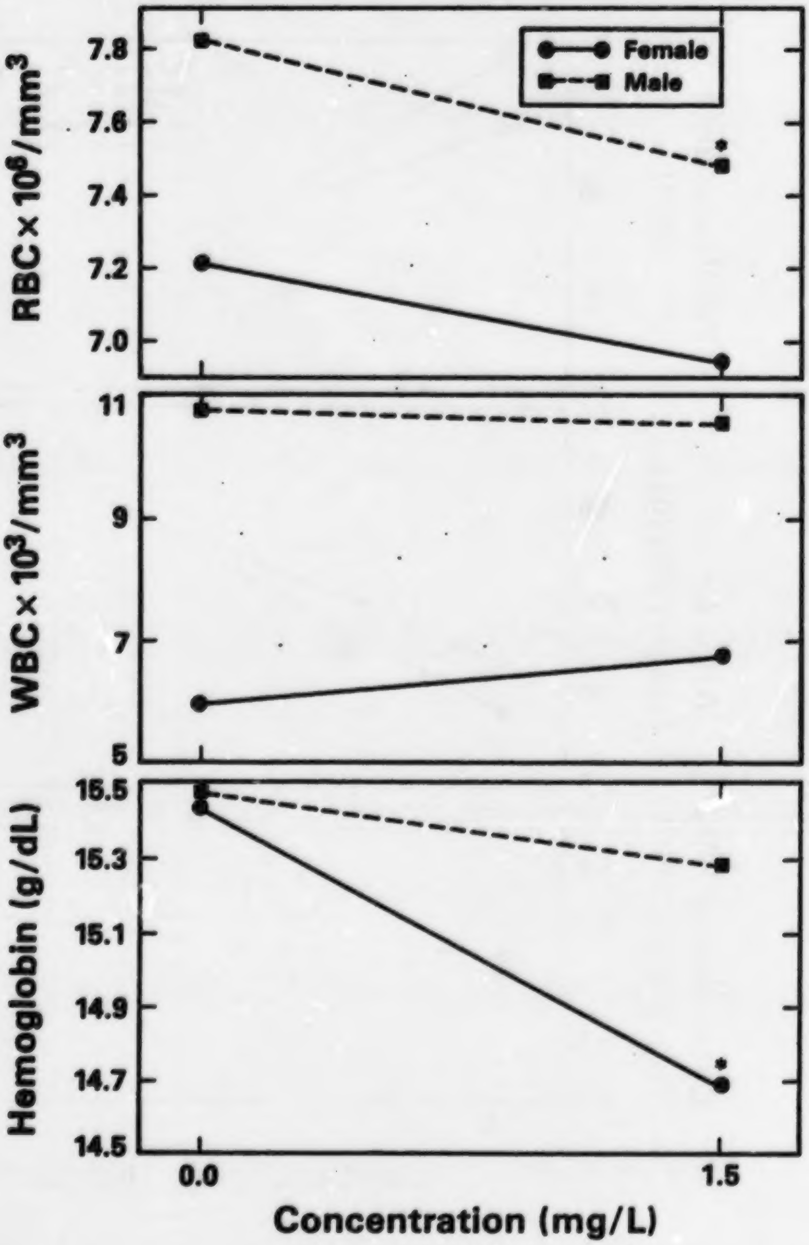

Figure 66. Comparison of female-male response to subchronic exposure on RBC, WBC, and hemoglobin. *Significantly different from control ($p < 0.05$).

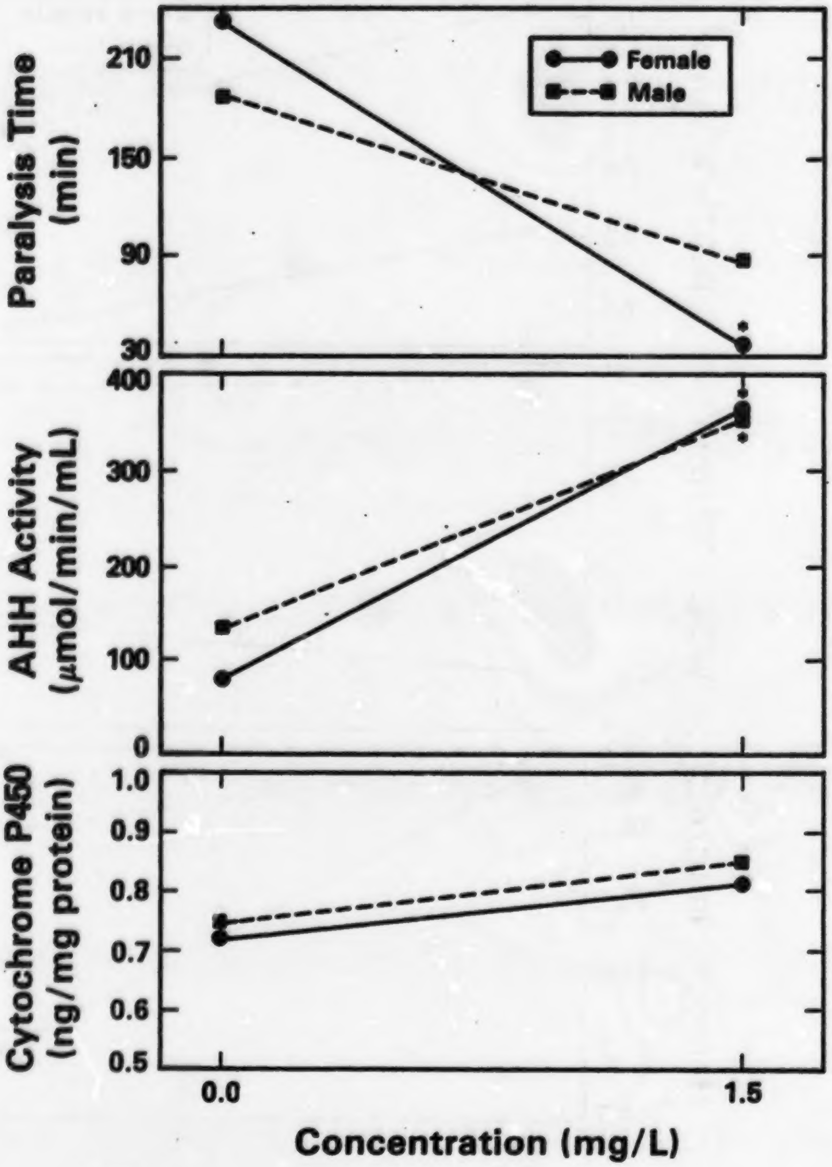

Figure 67. Comparison of female-male response to subchronic exposure on paralysis time, AHH activity, and cytochrome P450. *Significantly different from control ($p < 0.05$).

TABLE 16. SUMMARY OF PHASE III - SUBCHRONIC STUDY: PART C

	Pulmonary Response	
Parameter	Female	Male
Histopathology	Slight ↑ macrophages	Moderate ↑ macrophages
Body weight	Overall male effect	Overall male effect
Lung weight	↑ at 1.5 mg/L	↑ at 1.5 mg/L
Pulmonary physiology	Overall male effect	Overall male effect
Pulmonary edema	↑ at 1.5 mg/L	↑ at 1.5 mg/L
Pulmonary cells	↓ % macrophages ↑ % PMNs ↑ % lymphocytes	↓ % macrophages ↑ % PMNs
	Systemic Response	
Histopathology	Congestion of mandibular lymph nodes	Congestion of mandibular lymph nodes
Hematology	↓ MCV ↓ % hematocrit ↓ hemoglobin	↓ RBC
AHH activity	↑ at 1.5 mg/L	↑ at 1.5 mg/L
Zoxazolamine-induced paralysis time	↓ at 1.5 mg/L	NS[a]
Cytochrome P450	NS	NS

a. NS = no significant effects.

DISCUSSION

Based on the results of these studies, there appears to be both a pulmonary and systemic response in rats due to inhalation of fog oil smoke. Mortality studies demonstrated a steep mortality curve by both time and concentration. There was no difference between sexes as to mortality. The LC_{50} for fog oil smoke was determined to be 5.2 mg/L for 3.5 hr of exposure. Results were similar in the 4-wk subacute and 13-wk subchronic exposures with the extent of injury being concentration- and time-related.

Body weight decreases were observed during both subacute and subchronic exposures. This appeared to be the result of the fog oil smoke-exposed rats not eating during the days when exposures were occurring. Then, during the intervening days from one weekly exposure to another, the animals ate and regained some of the weight lost during exposure. It is therefore reasonable to conclude that increased fog oil smoke concentration and longer exposure times contributed to a decrease in normal weight gain over the exposure period. During the subacute studies, increasing the frequency of exposure did not affect body weight. The actual weight loss after the subacute exposures in the worst case situation was less than 10%. This difference appears to be due to an anorexigenic effect on the days when exposure occurred. During the subchronic studies, the weight loss in males was substantial (-72 g) at 1.5 mg/L and appears to be an indication of toxicity. The females also lost weight during the subchronic study (-10 g); however, the loss was not significant.

Male and female rats exposed to 0.5 or 1.5 mg/L of fog oil smoke for either 70 min or 3.5 hr/day, 4 days/wk for 4 wk, exhibited increased alveolar macrophage populations in the alveoli of their lungs. The cytoplasm of these macrophages contained eosinophilic, protein-like material. The treatment-related lesions were concentration dependent. The severity of this effect was slight to moderate in rats exposed to 1.5 mg/L and minimal to slight in rats exposed to 0.5 mg/L. In addition, multifocal pneumonitis was observed in some male rats exposed to 1.5 mg/L, but no sign of pneumonitis was seen in female rats at this concentration. No treatment-related lesions were observed in the other tissues examined. After the subchronic exposures, treatment-related lesions were observed in both male and female rats. A concentration-related accumulation of macrophages was present in the alveolar lumen and sinusoids of the peribronchial lymph nodes of rats exposed at 0.2, 0.5, and 1.5 mg/L. The lesions observed after 1.5 mg/L exposure were still evident following the 4-wk recovery period. No lesions were observed in control animals. Congestion, focal hemorrhage, and multifocal granulomatous pneumonia were also observed in the male rats after 1.5 mg/L exposure. Some granulomas formed after 13 wk of exposure, but the majority were not observed until after the 4-wk recovery period. This occurrence suggests a progressive lesion after cessation of exposure.

Of the pulmonary physiology parameters studied, only lung wet and dry weights and EEV were significantly affected after 4 or 13 wk of exposure to

1.5 mg/L. None of the parameters showed significant effects at the 0.2 or 0.5-mg/L concentration. Because lung wet and dry weights were affected at the 1.5-mg/L concentration in the same manner, we believe this is due to an increase in lung cellularity, as verified by the pathology results. This effect was evident in both females and males, both immediately after exposure and after the recovery period. Because diffusing capacity was unchanged and normal gas exchange was maintained, we speculate that the significant increase in EEV at 1.5 mg/L observed during subacute exposure (4 wk) may be an attempt of the animal to compensate for a change in the alveoli, such as oil deposition or cell infiltration. Because it is not uncommon to observe increases in EEV following exposure to a respiratory irritant, the animal may have been responding to the exposure by taking shallower breaths.

The effect of fog oil smoke on pulmonary cells was supported by the lung weight changes and the histopathology results. Cell viability, total cell count, and cell differentials were measured. There was a significant increase in total cells following both subacute and subchronic exposure to 1.5 mg/L, which appeared to be due to an influx in PMNs. There was a 25% increase of alveolar macrophages following the 4-wk subacute exposure to 0.5 mg/L as compared to controls. However, these data are difficult to interpret because the control values are lower than historical control data from this laboratory. Yet, in the subchronic exposures to 0.5 mg/L there was also a significant increase in the percentages of PMNs and lymphocytes and a decrease in the percentage of macrophages indicating an inflammatory response. Rats exposed for 4 wk to 1.5 mg/L also showed an 8% increase in the percentage of eosinophils in the total cell population. However, due to heteroscedasticity and non-normal data problems, no biological significance should be attributed to these data. Viability of the pulmonary cells appeared to be enhanced by the subacute fog oil smoke treatment at both the 0.5- and 1.5-mg/L concentrations; however, there was a significant interaction between replicates. Effects were actually greater at 0.5 mg/L than 1.5 mg/L. The mechanisms responsible are unknown. It is possible that the fog oil smoke caused a lysis of resident nonviable cells, and/or the influx of new cells (primarily PMNs) leading to an apparently higher viability. Biologically important changes in lymphocyte populations were not noted after any exposure, although in some cases statistically significant increases were noted.

Protein levels in the lungs were measured as an indicator of pulmonary edema. We concluded from these studies that rats exposed to 0.2, 0.5, and 1.5 mg/L, for 4 or 13 wk, regardless of duration time or sex, showed an increase in protein content of the lung lavage fluid. This protein increase was probably due to leakage of serum proteins into the lung that accompanies edema.

Various extrapulmonary parameters were measured. The behavioral data reported (figure-eight maze response) lack a meaningful biological explanation for the patterns observed. It is most likely that the significant interaction reported was a statistical artifact and that the effects of the 4-wk exposures were either negligible or not detectable with this sample size. It was decided that further testing with such large numbers of animals was not practical; thus, this parameter was eliminated from further studies.

Clinical chemistry parameters were measured as both a toxicological indicator and quality control measure. Significant effects were seen most often at the 2 day/wk for 4-wk frequency, at the 3.5-hr time and 0.5-mg/L concentration. However, effects in the worst case exposure group are lacking and the data do not suggest any trends. The significant decrease in serum triglycerides after 13 wk of exposure to 1.5 mg/L of fog oil smoke was probably the result of dietary change as reflected in the weight loss reported. The amylase increase could be explained by pancreatic disease, but no histopathological evidence supported this theory. It is more likely that the ingestion of oil by preening caused a stimulation of the salivary glands, which in turn caused a release of amylase into the bloodstream. Thus, we must conclude that the fog oil smoke did not adversely affect the clinical chemistry parameters in any consistent trend. From a quality control point of view, this suggests homogeneity between the exposed and control groups.

Various hematological parameters were measured to determine the effects of fog oil smoke inhalation on the blood. In the subacute studies' worst case situation (highest concentration for the longest and most frequent exposure), there was a significant decrease in MCV that appeared to be compensated for by an increase (though not significant) in the number of RBC. In the subchronic studies, there were differences between female and male rats after 13 wk of exposure to 1.5 mg/L of fog oil smoke. The combined hematological effects observed in the female suggest the possibility of an anemic response. The significant decreases in MCV and in percentage of hematocrit are outside the lower boundary of normal for albino rats. The drop in hemoglobin was statistically significant but still within normal limits, as was the RBC. Further examination of hemotological parameters would be necessary to form a firm conclusion.

There appeared to be little to no immunological response to fog oil smoke. Mitogen response of spleen and WBC was negative, but there was some enhancement of NK cell activity after the subacute exposures. This suggested that the fog oil smoke was introducing new antigens in some way (possibly by alteration of host cell membranes), which subsequently stimulated NK cell activity. NK cell activity is thought to have a major role in immune surveillance. However, it is important to note that two different strains of rats were used for the 4-wk (Fischer 344) and the subchronic (CD) studies due to exposure logistics. Correlations should not be drawn between these data.

Four parameters of xenobiotic metabolism were measured: pentobarbital-induced sleeping time, zoxazolamine-induced paralysis time, cytochrome P450 concentration, and AHH activity. There was no reproducible change in sleeping time or liver cytochrome P450 due to fog oil smoke exposure. However, paralysis time and AHH activity were significantly decreased at 0.2, 0.5, and 1.5 mg/L of fog oil smoke (4 and 13 wk) in a concentration-response manner. The alterations in AHH activity and zoxazolamine-induced paralysis time but not cytochrome P450 or sleeping time suggests a selective effect on cytochrome P_1-450, which is responsible for BAP metabolism. This induction of AHH could be due to the polycyclic hydrocarbons in the fog oil smoke. Because these are extrapulmonary effects, the question arose as to whether the effects in the liver were due to ingestion of the fog oil smoke. As reported in the results, a comparison of whole-body to nose-only exposure showed no difference in paralysis time due to method of exposure. This confirms that the results were due to inhalation of the smoke and not to ingestion of the oil.

In conclusion, 4-wk subacute and 13-wk subchronic inhalation of fog oil smoke appeared to cause an inflammatory and edematogenic response in the lungs of male and female adult rats, yet pulmonary function and gas exchange were not significantly compromised. Formation of granulomas after the recovery period suggests a progressive lesion in the lung following the subchronic exposure in the male rats. Extrapulmonary effects consisted of altered xenobiotic metabolic mechanism, as suggested by a possible induction of a specific isoenzyme of the cytochrome P450 system.

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